**DISSERTATION**

*on*

# TYPING OF HEMOLYTIC ANEMIAS - ROLE OF BASIC DIAGNOSTIC SCREENING PANEL

*submitted in partial fulfillment of*
*requirements for*

**MD DEGREE EXAMINATION**

**BRANCH-III PATHOLOGY**

**THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**
**CHENNAI**


**TIRUNELVELI MEDICAL COLLEGE**
**TIRUNELVELI**
**APRIL 2016**

## CERTIFICATE

This is to certify that the dissertation titled **"TYPING OF HEMOLYTIC ANEMIAS- ROLE OF BASIC DIAGNOSTIC SCREENING PANEL",** is a bonafide work done by **Dr.D.VISHNUPRATHAP**, Post Graduate Student, Department of Pathology, Tirunelveli Medical College, Tirunelveli – 627011, in partial fulfilment of the university rules and regulations for the award of MD DEGREE in PATHOLOGY BRANCH-III, under my guidance and supervision, during the academic period from September 2013 to September 2015.

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## CERTIFICATE

I hereby certify that this dissertation entitled "**TYPING OF HEMOLYTIC ANEMIAS- ROLE OF BASIC DIAGNOSTIC SCREENING PANEL"** is a record of work done by **Dr.D.VISHNUPRATHAP**, in the Department of Pathology, Tirunelveli Medical College, Tirunelveli, during his postgraduate degree course period from 2013- 2016. This work has not formed the basis for previous award of any degree.

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## DECLARATION

I solemnly declare that the dissertation titled **"TYPING OF HEMOLYTIC ANEMIAS- ROLE OF BASIC DIAGNOSTIC SCREENING PANEL"** was done by me at Tirunelveli Medical College, Tirunelveli – 627011, during the period September 2013 to September 2015 under the guidance and supervision of **Prof. J. SURESH DURAI, MD**, to be submitted to The Tamil Nadu Dr. M.G.R. Medical University towards the partial fulfilment of requirements for the award of MD DEGREE in PATHOLOGY BRANCH-III.

Place : Tirunelveli

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PROTOCOL TITLE: Typing of Hemolytic Anemias – Role of Basic Diagnostic Screening Panel

NAME OF PRINCIPAL INVESTIGATOR: Dr. D.Vishnu Prathap
DESIGNATION OF PRINCIPAL INVESTIGATOR: Resident in Pathology
DEPARTMENT & INSTITUTION: Department of Pathology, Tirunelveli Medical College

*Dear Dr.* D.Vishnu Prathap, *The Tirunelveli Medical College Institutional Ethics Committee (TIREC) reviewed and discussed your application during the IEC meeting held on 28.12.13.*

**THE FOLLOWING DOCUMENTS WERE REVIEWED AND APPROVED**

| | | |
|---|---|---|
| 1. | TIREC Application Form | ☑ |
| 2. | Study Protocol | ☑ |
| 3. | Department Research Committee Approval | ☑ |
| 4. | Patient Information Document and Consent Form in English and Vernacular Language | ☑ |
| 5. | Investigator's Brochure | ☐ |
| 6. | Proposed Methods for Patient Accrual Proposed | ☑ |
| 7. | Curriculum Vitae of the Principal Investigator | ☑ |
| 8. | Insurance /Compensation Policy | ☑ |
| 9. | Investigator's Agreement with Sponsor | ☐ |
| 10. | Investigator's Undertaking | ☑ |
| 11. | DCGI/DGFT approval | ☐ |
| 12. | Clinical Trial Agreement (CTA) | ☐ |
| 13. | Memorandum of Understanding (MOU)/Material Transfer Agreement (MTA) | ☑ |
| 14. | Clinical Trials Registry-India (CTRI) Registration | ☐ |

**THE PROTOCOL IS APPROVED IN ITS PRESENTED FORM ON THE FOLLOWING CONDITIONS**

1. The approval is valid for a period of 2 year/s or duration of project whichever is later
2. The date of commencement of study should be informed
3. A written request should be submitted 3weeks before for renewal / extension of the validity
4. An annual status report should be submitted.
5. The TIREC will monitor the study
6. At the time of PI's retirement/leaving the institute, the study responsibility should be transferred to a person cleared by HOD
7. The PI should report to TIREC within 7 days of the occurrence of the SAE. If the SAE is Death, the Bioethics Cell should receive the SAE reporting form within 24 hours of the occurrence.
8. In the events of any protocol amendments, TIREC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. The PI must comment how proposed amendment will affect the ongoing trial. Alteration in the budgetary status, staff requirement should be clearly indicated and the revised budget form should be submitted.
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented.
   d. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IEC, only then can they be implemented.
   e. Approval for amendment changes must be obtained prior to implementation of changes.
   f. The amendment is unlikely to be approved by the IEC unless all the above information is provided.
   g. Any deviation/violation/waiver in the protocol must be informed

**STANDS APPROVED UNDER SEAL**

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## ABBREVIATIONS

| | |
|---|---|
| AHG | Anti human globulin |
| AIHA | Auto immune haemolytic anemia |
| APTT | Activated partial thromboplastin time |
| ATP | Adenine tri phosphate |
| BTT | Beta thalassaemia trait |
| CB | Conjugated bilirubin |
| CBC | Complete blood count |
| CCF | Congestive cardiac failure |
| CLL | Chronic lymphocytic leukemia |
| CVS | Cardio vascular system |
| DAT | Direct antiglobulin test |
| DIC | Disseminated intravascular coagulation |
| DNA | Deoxyribonucleic acid |
| DPX | Distrene plasticier xylene |
| EBV | Ebstein bar virus |
| EDTA | Ethylene diamine tetra acetic acid |
| FL | Femto litres |
| G6PD | Glucose 6 phosphate dehydrogenase deficiency |
| GLU | Glutamine |
| H2O2 | Hydrogen peroxide |
| HB A | Hemoglobin A |
| HB A2 | Hemoglobin A2 |
| HBC | Hemoglobin C |
| HBD | Hemoglobin D |
| HBE | Hemoglobin E |
| HBH | Hemoglobinh |
| HBS | Hemoglobin S |
| HCL | Hydrogen chloride |
| HE | Heriditary elliptocytosis |
| HP | Heriditary pyropoikilocytosis |
| HS | Heriditary spherocytosis |
| HSV | Herpes simplex virus |
| HUS | Haemolytic uremic syndrome |
| IAT | Indirect antiglobin test |
| ICMR | Indian council of medical reasearch |
| IGA | Immunoglobulin A |
| IGG | Immunoglobuling |
| IGM | Immunoglobulin M |
| IHA | Immune haemolytic anemia |
| KCL | Pottasium chloride |

| | |
|---|---|
| LDH | Lactate dehydrogenase |
| LYS | Lysine |
| MCH | Mean corpuscular volume |
| MCHC | Mean corpuscular hemoglobin concentration |
| MCV | Mean corpuscular volume |
| M RNA | Messenger ribonucleic acid |
| NACL | Sodium chloride |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate hydrogen |
| N RBC | Nucleated RBC |
| O2 | Oxygen |
| OF | Osmotic fragility |
| OFT | Osmotic fragility test |
| PBF | Peripheral blood film |
| PCV | Packed cell volume |
| PG | Pico gram |
| PNH | Paroxysmal nocturnal haemoglobinuria |
| PT | Prothrombin time |
| RBC | Red blood corpuscle |
| RDW | Red cell distribution width |
| RDW SD | Red cell distribution width standard deviation |
| RES | Reticuloendothelial system |
| RNA | Ribonucleic acid |
| RPM | Rotations per minute |
| SAO | South asian ovalocytosis |
| SCD | Sickle cell disease |
| SLE | Systemic lupus erythematosis |
| TB | Tuberculosis |
| TTP | Thrombotic thrombocytopenic purpura |
| UCB | Unconjugated bilurubin |
| VAL | Valine |
| WBC | White blood corpuscle |
| WHO | World health organisation |

# CONTENTS

# TYPING OF HEMOLYTIC ANEMIAS - ROLE OF BASIC DIAGNOSTIC SCREENING PANEL

**Abstract**

In the developing world haemolytic anaemia are becoming a huge disease burden. The diagnostic tests are time consuming and costly as well.in our study we subjected the patients with a clinical suspicion to basic panel of investigations to establish an underlying haemolytic process and tried to categorise haemolytic anemia. Our panel of simple investigations include CBC, peripheral blood smear, reticulocyte count, osmotic fragility test, serum bilurubin, hemoglobinuria and hemosiderinuria. With this panel we were able to diagnose50% of cases as thalassaemia, 21.42% of caeses as post haemolytic state,7.145 as haemolytic disease of new born and the remaining as others in which an underlying haemolytic process was established.

**Key words :**

haemolytic anemia, thalassaemia, post haemolytic state, hemosiderinuria ,peripheral smear

# INTRODUCTION

Haemolytic anaemias account for a huge disease burden, particularly in the developing world. And it is on the increase as more and more diseased children now routinely survive into adulthood. Proper diagnosis and management are of paramount importance.

Investigation of haemolytic anaemia involves three steps.

1. Establishing that it is haemolytic anaemia
2. Making a working diagnosis necessary for clinical management
3. Special investigations for sub-typing, genotyping etc,

Haemolytic anaemia is a condition of decreased red cell survival in which the intact regenerative capacity of marrow is unable to compensate for the accelerated loss. When the production keeps pace with the destruction it is compensated haemolytic state. It follows that, for establishing haemolytic anaemia we have to gather evidence of increased destruction and also increased production.

Red cell survival and kinetic studies are the definitive means of establishing haemolytic anaemia. However for all practical purposes, Anaemia, increased unconjugated bilurubin and reticulocytosis are diagnostic. While the first two indicate red cell destruction the third is indicative of increased production. Increased urobilinogen in urine and the presence of polychromatic red cells in the smear are other useful and simple indicators.

Splenomegaly is found in chronic cases, sickle cell anemia being an exception in that the spleen usually shrinking this disease due to infarctions. Widening of dipole skull and facial bones during childhood gives rise to typical features often described as haemolytic facies. This is most marked in thalassemia major.

In India with majority of people in low socioeconomic strata, complete evaluation of haemolytic anaemia will be of huge burden. A basic panel of tests will be of immense use to classify or type haemolytic anaemia and directions for further work up. The basic panel of tests like CBC, peripheral smear study, serum bilurubin, hemoglobinuria, and hemosiderinuria will establish the diagnosis of haemolytic anaemia and further aids in directions for specialised investigations like haemoglobin electrophoresis, genetic and molecular studies. Osmotic fragility test will be of definite use in diagnosing diseases like hereditary spherocytosis, thalassaemia and sickle cell anaemia etc. along with other parameters like CBC and peripheral smear. Majority of Immune haemolytic anaemias can be diagnosed using coomb's tests (direct and indirect).

# Aims and objectives

1. To formulate a panel of basic screening tests for evaluation of haemolytic anaemia

2. To assess the age and sex distribution of various type of haemolytic anaemia

3. To assess the incidence of various types of haemolytic anaemia

# REVIEW OF LITERATURE

Haemolytic anemias can be clinically classified into hereditary and acquired. It can be classified based on the pathophysiology into intracapsular and extracapsular. In general intracapsular haemolytic anemias in which the defect is to be found in the red cell are hereditary whereas paroxysmal nocturnal hemoglobinuria is an exception. On the other hand, the extracapsular haemolytic anemias are almost always acquired.

More than 95% of all symptomatic haemolytic anemias belong to a handful of entities in which the prevalence of chronic diseases like thalassemia and sickle cell anaemia would be higher. There are millions of people with sickle cell anaemia and their number are in increasing trend as life expectancy improves with better medical care. G6PD despite the vast number of people having mutant genes poses problems only in a few.

Table-1. Classification of Haemolytic anemias [1]

| | **Intracorpuscular defects** | **Extracorpuscular factors** |
|---|---|---|
| Hereditary | Hemoglobinopathies | Familial haemolytic uremic syndrome |
| | Enzymopathies | |
| | Membrane-cytoskeletal defects | |
| Acquired | Paroxysmal nocturnal hemoglobinuria | Mechanical destruction (macroangiopathic) |
| | | Toxic agents |
| | | Drugs |
| | | Infectious |
| | | Autoimmune |

Hemoglobinopathies include thalassaemias and sickle cell syndromes. Enzymopathies incorporates G6PD (glucose -6-phosphate dehydrogenase) and pyruvate kinase deficiencies. Membrane cytoskeletal defects are hereditary spherocytosis, hereditary elliptocytosis, south asian ovalocytosis and pyropoikilocytosis. The cumulative gene frequency of hemoglobinopathies in India lies nearly to 4.2 per cent. This disease prevalence varies with the geographic locations and racial populations. The research carried by The Indian Council of Medical Research (ICMR) revealed six cities of six states results, and have worked out that the overall incidence of beta thalassemia trait (BTT) was 2.78 per cent.[2] In another study from different parts of India have shown an incidence of beta thalassemia to be 3-15 per cent.[3-9]. In Orissa[7] HbS is very common whereas in West Bengal the commonest hemoglobinopathy seen is HbE[8,9] and thus this disease incidence also differs in different parts of India. Whereas in Punjab (Hb D-Punjab) shows greatest prevalence among Sikhs (2 %).[9] The ICMR study showed that HbE was mainly seen in Assam (23.9 per cent) and Kolkata in West Bengal (3.92 %).[3] It is well established that the incidence of HbE gene in the North Eastern region of India is one of the highest incidence worldwide.[11] Different states of the North Eastern region show a variable incidence of HbE varying from 16.2 per cent to 47.3 per cent.[12-14] A huge migrant tea garden population also shows a high incidence of HbS.[14],[15]Hb E disease will present as variable clinical phenotypes viz. homozygous, heterozygous or compound heterozygous forms.

The thalassaemias are a heterogeneous group of inherited disorders of haemoglobin characterised by reduced or absent production of one (or rarely more) of

the globin chains. They are the commonest single gene disorders in the world. The thalassaemias constitute a major public health problem in the countries surrounding the Mediterranean and in the Middle and the Far East. Lack of standard medical care and of regular and safe blood supply in some countries is associated with profound morbidity and mortality from thalassaemias. This is one of the prevalent monogenic disorders in the Indian subcontinent. It is estimated that there are 30–40 million carriers, and 8,000 to 10,000 thalassaemics are born every year in India.[2] In contrast to the global frequency of 1.5 % , the average carriers of β-thalassaemia trait (BTT) in India comprise 3.3 per cent of the population. Though its presence is recorded from different regions of India, the frequency varies widely between 0.5 and 17 per cent in different geographical regions [2]. Unlike β-thalassaemia, the distribution of sickle cell anaemia (sickle cell haemoglobin (HbS) is restricted to specific geographic regions. Two features are obvious from the studies done in northern India and North Eastern states on α- and β-globin traits in India: the frequency of traits varies with region and there is a significant gap in knowledge from regions like, Rajasthan, Bihar, Jharkhand, Tamil Nadu, Kerala,UP etc. that comprise massive Indian population.

Sickle cell syndromes are present throughout the world. It has a high morbidity and mortality index. It affects multiple organs because of the vasoocclusive phenomenon.

In India, sickle cell trait is found in the Veddian tribes[19], the tribals of Western India[20] and the labour tribes drawn from Orissa working in the Assam tea gardens has

been reported. It is also present in a group of marathy speaking people of low socio economic strata and in the people of Nagpur and surrounding districts.

First description of inherited disorder of hereditary spherocytosis was made in the year 1871 and it was followed by the first splenectomy. In Northern Europe and North America the commonest etiology for chronic hemolysis is HS. The incidence being 1 in5000 births .[21] HS occurs in all races but has a high prevalence in people of Northern European descent (1:5000). In our country, HS is found mainly in North India.

Hereditary Eliptocytosis (HE**)** affects 3-5 per 10,000 people in the United States[22.] It is prevalent in people of Mediterranean and African desendents. In India, its frequency is highest among Sikhs of Punjab (3.6%).it is of low frequency in Jammu & Kashmir (3.3%) and UP (2.3%). [23-25] HPP is most often found in those of African desendents, whereas spherocytic HE is most often found in Caucasians, HE seems to confer protection against malaria, particularly the SAO subtype, and its prevalence can be much greater in areas of the world where malaria is endemic. The majority of forms are transmitted in an autosomal dominant fashion except for HPP, which is autosomal recessive.

HE characteristically present as oval or elliptical shaped RBCs on the peripheral blood film. Mutations in membrane skeletal proteins that disrupt lateral interaction and contacts with the overlying lipid bilayer are unaffected (unlike in hereditary spherocytosis) most commonly, the mutations are in spectrin and result in the inability of spectrin dimers to form oligomers. Mutations of alpha spectrin, beta

spectrin and protein 4.1 are responsible for 65%, 30%, and 5% of cases of hereditary elliptocytosis respectively.[26]

There are five major subtypes *viz.*, silent carriers, common HE (90 %), hereditary pyropoikilocytosis (HPP), spherocytic HE (10 %), and South Eastern Asian ovalocytosis (SAO).

Immune haemolytic anaemia (IHA) is an acquired type of haemolytic anemia. The lysis of RBC occurs due to adherence of antibodies to the antigens present over the surface of RBC. Red cell lysis is mediated by the complement system and macrophages OF RES.

There are three types of immune haemolytic anemia; [27]

1.Auto immune

2.Aalloimmune

3. Drug induced

In Caucasians the incidence of IHA is 1 in 80,000 to 100,000 of per year.[28] The peak incidence of age is between 60 and 70 years. Females are most commonly affected (60%).[29]

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a hereditary intrinsic enzyme defect, with about 400 million people affected worldwide and is characterised by reduced activity of G6PD red cells, and haemolysis occurrs after exposure to oxidant stress. More than 400 biochemical variants of G6PD have been identified. The variants are grouped into five classes by World Health Organization Scientific Working Group. Polymorphic mutations occur with high frequency in malaria-endemic areas (WHO class II and III) and include G6PD A (common in

Africa) and G6PD Mediterranean (common in Mediterranean countries, Middle East, and India). In such cases, haemolysis develops only following oxidant exposure. Sporadic mutations occur anywhere in the world at low frequency and patient develops chronic haemolytic anaemia (WHO class-I). The well-known abnormal G6PD variants associated with G6PD deficiency are G6PD A (prevalent in Africa; half life 13 days) and G6PD Mediterranean (half life several hours). G6PD variant with normal enzyme activity is G6PD B (half life 60 days). The deficient variants common in India are namely Mediterranean, Kerala-Kalyan, and Orissa. India has a diverse population and a recent review summarized G6PD prevalence across the country as 0–10 per cent. The prevalence of 6.17 % is concurrent with the prevalent rate in India. [30,31]

In males by using biochemical screening methods the prevalence of G6PD deficiency was calculated. It was found to be in the range of 0–65 %. The Vataliya Prajapati community has got the highest incidence and the prevalence is also high in South and Western India. [30]

**Thalassaemias**

Classification of Thalassaemias

The classification of thalassaemias is based on the type of globin chain that is deficiently synthesised, or clinical expression of the disease.

Classification according to the type of globin chain which is deficiently synthesised:

The two most common types are α (alpha) and β (beta) thalassaemias. Less common types are *δβ* (delta beta) thalassaemia and γδβ (gamma-delta-beta) thalassaemia. Classification according to clinical severity: β thalassaemias have been

clinically classified on the basis of severity of anaemia into three types *viz.*, thalassaemia major, thalassaemia intermedia, and thalassaemia minor. Patients with severe transfusion dependent anaemia are said to have thalassaemia major. In thalassaemia minor, affected individuals are usually asymptomatic with mild or no anaemia in spite of prominent red cell abnormalities in peripheral blood. Thalassaemia intermedia is characterised by intermediate degree of severity of anaemia that does not require regular blood transfusions. Each of these clinical types are genetically diverse. Clinical types of α thalassaemia are Hb Bart's hydrops foetalis syndrome, HbH disease, α thalassaemia trait and silent carrier.

β-Thalassemias

β-Thalassemias are disorders caused by reduced synthesis of β-globin, encoded by the β-globin locus on chromosome 11. There are about 200 distinct causative mutations. Of these about 28 mutations have been documented in Indian patients, [32-34]; 80 % of cases can be attributed to 20 relatively common mutations. Most mutations are single-base substitutions that decrease the activity of the β-globin promoter activity or have deleterious effects on RNA processing/splicing/ translation .[32] Deletions (common in α-thalassemia) are rare. Individuals inheriting one defective β-globin allele typically have β-thalassemia major. Individuals inheriting two defective β-globin alleles have β-thalassemia major (Cooley anemia) or β-thalassemia intermedia, depending on the combination of alleles that are inherited.

**α thalassaemias**

α-Thalassemias are disorders caused by reduced synthesis of α-globin chains that are generally due to inherited α-globin gene deletions. Normally, each copy of

chromosome 16 contains two closely linked α-globin genes, such that there are four α-globin genes in the normal diploid state. Different forms of α-thalassemia of increasing severity are caused by deletions that result in the loss of one to four α-globin loci.[35]α-thalassemia minima with loss of one α-globin locus with virtually no clinical or laboratory findings. α-thalassemia minor with loss of two α-globin loci presents with microcytosis, hypochromasia, and mild anemia. HbH disease with loss of three α-globin loci marked by the presence of β4 hemoglobin (hemoglobin H, composed of a β-globin tetramer). Presents in infancy with jaundice, splenomegaly, and moderate microcytic hypochromic anemia.[36]Hydrops fetalis with loss of four α-globin loci results in death *in* utero or immediately after birth. Most haemoglobin consists of Hb Barts, a tetramer composed of fetal hemoglobin (γ4) that has such high $O_2$ affinity that it releases little $O_2$ to tissues.[37]

**Hemoglobin C disease**

This hemoglobinopathy is caused by HbC. It is produced by a mutation that results by shifting of glutamic acid with lysine at sixth position of β-globin. HbC is prone to polymerize into characteristic crystals and also results in increase of potassium or chloride co-transport, leading to cellular dehydration and slightly decreased red cell survival HbC trait is asymptomatic without anaemia. HbC disease is often asymptomatic but can result in mild hemolytic anaemia and splenomegaly. Compound HbC/HbS heterozygosity produces a sickling syndrome. It has that fall in between the entities namely sickle cell trait and sickle cell disease.

**Hemoglobin E disease**

It is caused as result of glutamic acid to lysine substitution at twenty sixth position of of beta-globin,[38]which creates an alternative splice site, when this splice site is used, an abnormal unstable mRNA is produced, resulting in a net reduction in β-globin synthesis and a thalassemia-like phenotype.

**Sickle cell syndromes**

The sickle cell syndromes are a group of haemolytic anemias which are due to a mutation in the beta-globin gene. The glutamic acid at the sixth position is switched with valine. When deoxygenation occurs haemoglobin S $\alpha 2\beta 2^{(6Glu \rightarrow Val\ 20)}$ undergo sickled shape.[17,18]

Because of the sickled shape the cells while passing through small capillaries and venules will stick to the endothelium. This will lead to vaso-occlusion and hemolysis. The spleen is the site of destruction of abnormal RBCs. This vaso-occlusion is cause for ischaemic episodes and end organ damage.[18]

The clinical manifestations of sickle cell syndromes are primarily due to vaso-occlusive episodes. These vaso-occlusive episodes can happen in any one of the organ systems like brain, kidney, lung etc. Patient will be presenting with excruciating pain pertaining to the affected organ (painful crises). There are multiple patterns of inheritance of HbS i.e a hemoglobinopathy and from one parent and HbC ($\alpha 2\beta 2^{6Glu \rightarrow Lys)}$ another parent.

Affected individual will have a haemolytic picture, with a PCV of 15–30 % and reticulocytosis. Initially, it was thought that anaemia will counteract the

vasoocclusion by reducing blood viscosity. Granulocytosis is common. Fluctuation of total WBC count is seen and it can be unpredictable during episodes of acute illnesses. The manifestations of vaso-occlusion are diverse. Sickle cell anaemia can be diagnosed based on the peripheral blood film with other laboratory parameters supporting an underlying haemolytic process viz, reticulocytosis, increased UCB and decreased serum haptoglobin etc along with intermittent episodes of ischemic pain.

**Hereditary spherocytosis**

Hereditary spherocytosis (HS) is a congenital haemolytic disorder characterised by an inherited defect in the RBC membrane cytoskeleton and causes the formation of spherocytic red cells.[21] Spherocytes are less deformable than normal red cells. So they are trapped and engulfed by the spleenic macrophages. Spherocytes are osmotically fragile. They have reduced surface area to volume ratio. Although most common mode of inheritance is autosomal dominant, autosomal recessive transmission occurs in some cases.

Normally, lipid bilayer of the red cell membrane is anchored to the underlying skeleton by two major linkages. The first linkage involves interaction of ankyrin with spectrin in skeleton and band 3 in the bilayer. The second attachment between skeleton and bilayer is provided by glycophorin C and protein 4.1. Deficiency in any of these interactions causes weakening of contact between lipid bilayer and skeleton. As a result, areas of the lipid bilayer, which are not directly supported by the underlying skeleton, are lost from the cells in the form of small lipid vesicles. This causes decrease in surface area of red cell relative to volume with resultant spherocyte

formation. HS may result from deficiency of following skeletal proteins—spectrin [39] ankyrin [40], band 3, and protein 4.2. The deficiency of Ankyrin is more common.[41] Recently it has been shown that the outcome of splenectomy depends on the cytokeleal membrane protein affected (whether predominantly spectrin/ankyrin, or band 3).[42]

**Hereditary Eliptocytosis (HE)**

HE spans a group of disorders that show oval or elliptical-shaped red blood cells on the peripheral blood film. It is caused by mutations in membrane skeletal proteins that disrupt lateral interaction; contacts with the overlying lipid bilayer are unaffected (unlike in hereditary spherocytosis). Mutations of alpha spectrin, beta spectrin and protein 4.1 are responsible for 65%,30%, and 5% of cases of hereditary elliptocytosis respectively.[26]

**IMMUNE HEMOLYTIC ANEMIA**

Haemolysis due to immune mechanism occurs when antibody and/or complement bind to red cell membrane. In immunologically mediated haemolysis, destruction of red cells usually occurs by type II (cytotoxic) hypersensitivity reaction. The antigen is on the surface of the RBCs. The specific antibody in the circulation binds with the antigen. This causes extravascular or intravascular red cell destruction.

Alloimmune hemolytic anemia requires previous senstisisation to allogenic RBCs, which may be due to pregnancy, blood transfusion or transplantation. The alloantibodies are non reactive towards autologous red cells. Drug-induced antibodies are capable of recognising either intrinsic red cell antigens or the drugs bound to red

cells. It is difficult to differentiate between antibodies that react with intrinsic RBC antigens and the autoantibodies.[27] The bound antibody and the target antigen are directly proportional to the degree of haemolysis. IgM antibodies are responsible for lysis of red cells by the classical complement pathway and than the IgG antibodies.[43-45]

By means of some trigger mechanism an autoantibody is synthesized which will bring about an accelerated clearance of RBC lysis which will not be effectively compensated by feedback mechanisms body resulting in haemolytic anemia. T cell regulation of B cells will not be able to detect autoantiboby. The autoantibody escapes in such a way that it makes subtle alteration in antigenic structure.[45] Genetic mutations, viral and bacterial infections, various inflammatory disorders, drugs, lympho-proliferative disorders etc., leads to the production of autoantibodies.[46-48].

Classification of AIHA is based on thermal characteristics of the antibody and presence or absence of underlying disease. In AIHA, autoantibodies may be of IgG, IgM, or IgA class. Generally, IgG and IgM antibodies are respectively of warm and cold types; however in paroxysmal cold haemoglobinuria, IgG antibodies are of cold-reactive type. Warm autoantibodies act avidly around 37°C and exhibit lower reactivity at a redued temperature. Cold autoantibodies react more vigorously around 0-4°C and generally show less reactivity at physiologic temperature. Some patients have both type of antibodies which is a very rare occurrence .[27]

## MICROANGIOPATHIC HAEMOLYTIC ANAEMIA

This refers to haemolytic anaemia resulting from intravascular fragmentation and lysis of red cells due to alteration in small blood vessels. Usually direct damage to red cells occurs when they pass through the fibrin strands deposited in the microcirculation. Common causes of microangiopathic haemolytic anaemia are Thrombotic thrombocytopaenic purpura (TTP), Haemolytic uraemic syndrome (HUS), Disseminated intravascular coagulation (DIC), malignant hypertension, Eclampsia and Disseminated malignancies. Severity of anaemia is variable. The characteristic feature on peripheral blood smear is fragmented red cells or schistocytes**.** Schistocytes include small red cell fragments with 1 to 3 sharp spicules as well as large helmet-shaped red cells from which fragments have been split off. Evidence of intravascular haemolysis and consumptive coagulopathy is often present.

### March haemoglobinuria

Intravascular haemolysis may result following physical exercise such as marching or running on a hard surface for prolonged period (e.g. long-distance runners). Traumatic destruction of red cells occurs within vessels of the feet. The usual complaint is of passing reddish-brown urine following physical exertion. Haemoglobinuria is transient in most cases. The degree of haemolysis is usually mild and does not cause anaemia.

### Cardiac haemolytic anaemia

Prosthetic cardiac valves may be associated with chronic intravascular haemolysis. This may be related to turbulent blood flow resulting from leaking valve

and presence of artificial surface in bloodstream. Degree of anaemia depends upon severity of mechanical damage to red cells.

**Haemolytic anaemia due to direct action of physical, chemical, or infectious agents**

**Physical agents**

Acute intravascular haemolysis follows extensive burns and its degree depends on body surface area involved. Haemolysis usually occurs within 1 to 2 days of burn injury and is due to direct action of heat on red cells.

**Chemical agents**

Haemolysis can occur due to direct toxic action of some chemicals such as lead, arsenic chloride (workers in galvanising or soldering industries), distilled water (introduction of large quantity of distilled water in circulation may follow intravenous injection or irrigation during surgery), and some insect venoms (spider).

**Infectious agents**

**Malaria**

Malaria is one of most common infectious cause for haemolytic anaemia. It is caused by the following four species viz. Plasmodia: P. vivax, P. falciparum, P. malariae, and P. ovale. Infected female Anopheles mosquitoes transmit the disease. Recurrent high grade fever with chills and rigors, and splenomegaly are the characteristic features. Icterus may be present. In P. vivax infection fever occurs on alternate days, while in P.falciparum infection it occurs daily.

**Problems with diagnosis of haemolytic anemias**

Clinical features of haemolytic anaemia are varied and can be attributed to anaemia, the compensatory response by the host, previous therapy and the primary pathology causing it. Patients may be asymptomatic for a long duration of time even with a minimal but a chronic haemolytic process. Clinical manifestations may include easy fatigability tachycardia, dyspnea, and angina. Chronic continued haemolytic process leads to the development of bilirubin stones in the gall bladder and such patients complain of abdominal pain. Bronzing of skin and diabetes are seen in haemosiderosis; iron overload may occur in those who receive repeated transfusions or those who have been underwent erroneous iron therapy. Dark urine may be due to presence of haemoglobinuria as well as hemosiderinuria. Patients with thrombotic thrombocytopenic purpura (TTP) may experience elevated body temperature, neurologic signs, kidney failure, and low platelet count along with symptoms of haemolysis. Leg ulcers may develop in patients with sickle cell anaemia and other haemolytic disorders due to reduced red blood cell (RBC) deformability and alterations of endothelial function.

On examination an individual with haemolytic anaemia may show signs of anaemia and haemolytic process along with symptoms of primary pathology. Generally patients present with generalised signs of anaemia like pallor of skin and palpebral conjunctiva. But they may not specific for the underlying haemolytic process. Increased heart rate, increased respiratory rate, and fall in blood pressure are due to anoxia and decreased blood pressure that can occur in severe anaemias but they are also not specific for haemolytic anemias. Jaundice may occur because of a slight

increase in UCB and the rise is not indicative of haemolytic disorders and can also be seen in hepatic disease and obstruction of the biliary system. Bilirubin levels rarely exceed 3 mg/dL in hemolysis, unless complicated by hepatic disease or gall bladder stones. Enlarged spleen can be seen in hereditary spherocytosis and other haemolytic disorders, but not in all. For instance, splenomegaly usually is absent in G6PD deficiency. The occurrence of splenomegaly could suggest a hidden disorder such as chronic lymphocytic leukemia (CLL), some lymphomas, or systemic lupus erythematosis (SLE). Malar rash and polyarthritis could also suggest SLE. Enlarged lymph nodes along with splenomegaly is consistent with CLL. An enlarged spleen may not always be not evident on physical examination and imaging may be necessary to define spleen size. Tenderness in right hypochondrium may indicate cholelithiasis (pigment gallstones) or other gallbladder diseases. Tachycardia and dyspnea may be evident when there is severe anaemia with an abrupt onset of haemolysis such as in AIHA. Angina pectoris and symptoms of CCF may indicate a cardiovascular pathology.

In all types of haemolytic anaemia evidence of heemolysis is appreciated by increased LDH, increased levels of UCB, decreased haptoglobin levels, and increased reticulocyte counts. But none of these indices are specific and with these parameters it is difficult to distinguish among varied etiology of haemolytic anemias. In routine clinical scenario the practical approach will be to differentiate between extravascular haemolysis i.e destruction by monocyte-macrophage system (splenic and hepatic) and the intravascular haemolysis i.e haemolysis occurring inside the blood vessels which may be antibody mediated.

Generally, anaemia due to red cell intrinsic factors and immune mediated etiology are extravascular. Microangiopathic haemolytic anaemia, infection associated and chemical induced haemolytic anemias are intravascular. The following flow charts provide a practical approach to investigate both intravascular and extravascular haemolytic anemias.[99]

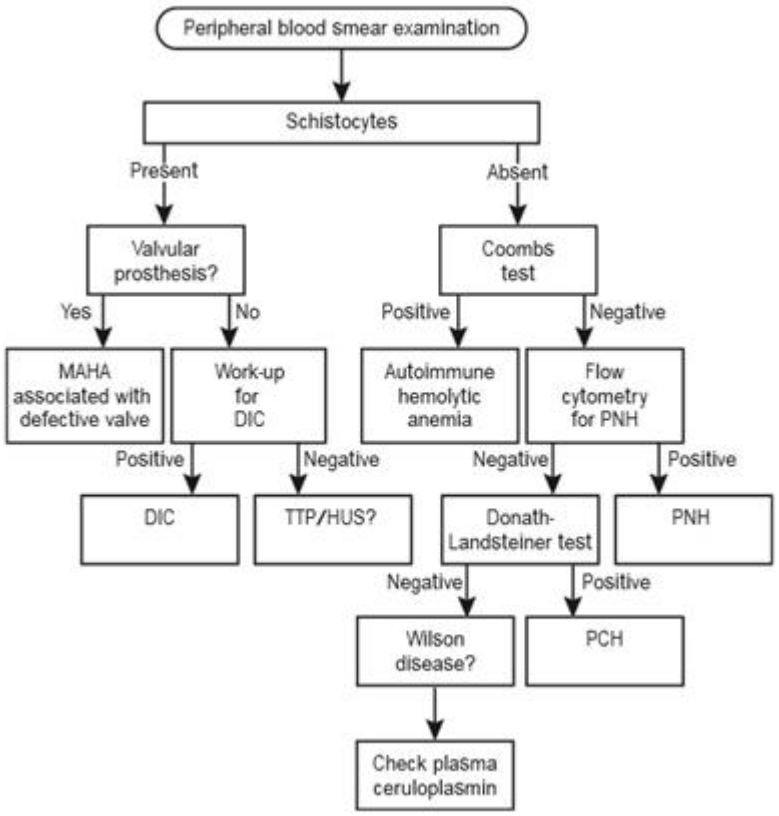



Figure 1. Evaluation of intravascular hemolysis. DIC = disseminated intravascular coagulation; HUS = hemolytic uremic syndrome; MAHA = microangiopathic hemolytic anemia; PCH = paroxysmal cold hemoglobinuria; PNH = paroxysmal nocturnal hemoglobinuria; TTP = thrombotic thrombocytopenic purpura.

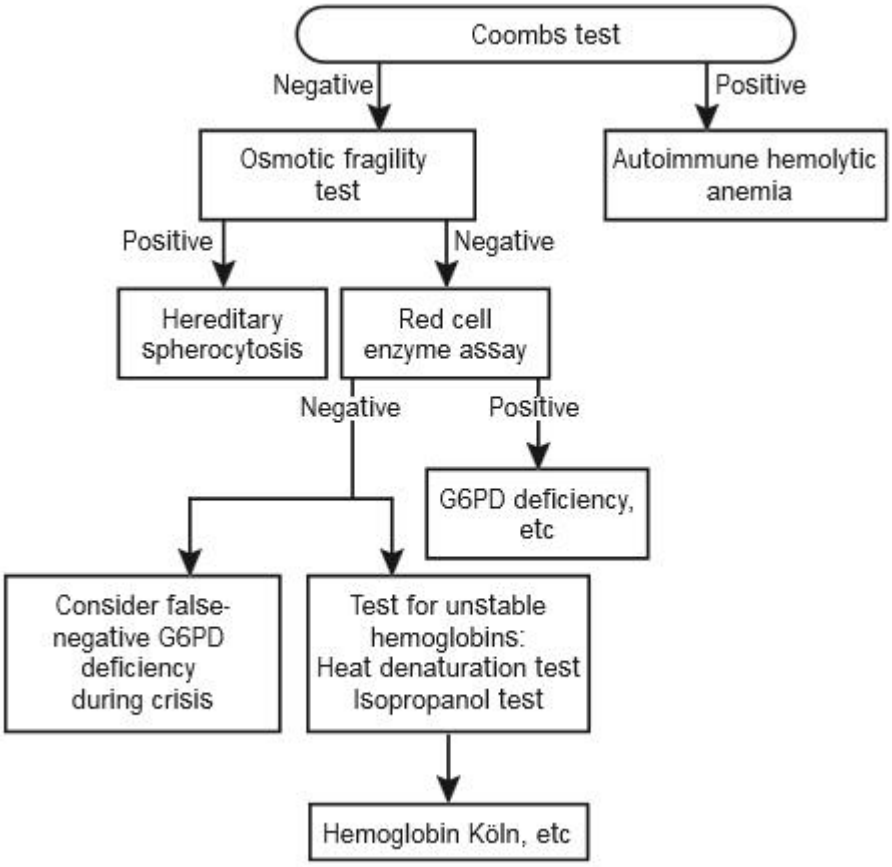



Figure 2. Evaluation of extravascular hemolysis. G6PD = glucose-6-phosphate dehydrogenase.

Variations in the Lactate Dehydrogenase levels and serum haptoglobin levels are the most sensitive indicators of haemolysis. The UCB level is not always has a positive correlation with haemolysis

Further laboratory evaluation may be directed by clinical history, clinical examination, peripheral smear examination and other laboratory findings. Ultrasonography is used to assess the spleen size, since the physical examination occasionally cannot always detect splenomegaly. Chest X ray, electrocardiography (ECG), and other studies are used to assess cardiopulmonary status.

**RBC indices**

Microcytic hypochromic anemia of iron deficiency will have a low MCV, and low MCH along with high RDW. This is because of low iron stores that can be confirmed by iron studies that may be seen in chronic intravascular haemolysis as well. In a haemolytic anaemia such as HS there will be ↑ MCV along with MCH and MCHC. The increased demand for folate in chronic haemolysis may lead to megaloblastosis and an increased MCV. RDW will reflect anisocytosis that has a varied significance in diagnosing haemolytic anaemias.

**Reticulocyte count**

An elevated reticulocyte count represents good compensatory response by the host to the anaemia irrespective of etiology whether haemolytic or non haemolytic. On the other hand, decreased reticulocyte count does not rule out a haemolytic process or it may be imperative that the host compensatory response is not adequate. Decreased reticulocytic count may be seen in marrow suppression due variety of causes in spite of an ongoing haemolytic process.

Thrombocytopenia is observed in a variety of haematological disorders. It is seen in autoimmune disorders like SLE, haematological malignancies such as CLL, and in a varied microangiopathic etiology. In Evans syndrome DAT test will be positive with low platelet counts.

Features on the PBF can help in the diagnosis of an underlying pathology associated with haemolysis. For example, presence of spherocytes on a peripheral

smear may suggest a haemolytic disorder which may be correlated with other RBC indices, the occurrence of schistocytes (fragmented red blood cells) suggests thrombotic thrombocytopenic purpura, haemolytic uremic syndrome or mechanical damage or a haematological malignancy such as CLL are characterized micro spheroytes by an abundance of small lymphocytes and smudge cells along with polychromatophylic RBCs may mimic a haemolytic disease. Polychromasia indicates RBC immaturity, which is an indicator of marrow response to an underlying haemolytic pathology.

**Serum LDH**

Elevated levels of serum LDH is one of the most sensitive indicators of an underlying haemolytic process. LDH elevation is sensitive for haemolysis, but not specific since LDH can be derived from any cell undergoing damage such as hepatocytes, tumor cells or from any other damaged viscera. LDH isoenzymes 1 and 2 are specific for RBC destruction. These enzymes are seen elevated in myocardial infarction as well.

**Serum Haptoglobin**

Decreased serum haptoglobin level is another sensitive indicator in a haemolytic process. The level of serum haptoglobin has a positive correlation with intravascular haemolysis than the extravascular one. The levels can be normal or elevated even with significant haemolysis in patients having infections or other reactive states.

**Unconjugated bilirubin (UCB)**

Unconjugated bilirubin though not a specific for hemolysis it is found elevated in majority of haemolytic anemias. The level of UCB rarely exceeds 3 mg/dL in haemolysis and higher levels should raise the suspicion of compromised hepatic function or cholelithiasis.

The direct Coombs test (DAT) is usually positive in autoimmune hemolytic anemia (AIHA). Though a negative DAT does not rule out a AIHA. About 5-10% of DAT negative coomb's had been reported.[49] The polybrene test, IRMA (immunoradiometric assay) and red blood cell–bound IgG are the other tests used to evaluate DAT negative individuals. [50] Micro RNA analysis has been found to aid in the diagnosis of AIHA in patients with CLL[52] Either myoglobinuria or porphyria may also result in dark urine and hence the urine should be tested for free hemoglobin. Hemoglobinuria occurs when the amount of free hemoglobin released during haemolysis is more than the available haptoglobin. Hemosiderin in the patients urine may indicate severe or intravascular haemolysis. The source of urinary hemosiderin is hemoglobinuria that occurs in severe and intravascular haemolysis and it is detected in iron-stained urinary sediment in the renal epithelial cells that have been shed. When hemoglobin in urine is reabsorbed by renal tubular cells, it is processed to hemosiderin. Therefore, urinary hemosiderin reflects hemoglobinuria and suggests severe or intravascular haemolysis.

Diagnostic difficulties with hereditary spherocytosis

The classic laboratory features of hereditary spherocytosis (HS) include the following [53,54]

1. Mild to moderate anaemia
2. Reticulocytosis
3. Increased Mean corpuscular haemoglobin concentration
4. Spherocytes on the peripheral blood smear
5. Hyperbilirubinemia
6. Abnormal osmotic fragility test

In HS peripheral blood smear shows spherocytes which are uniform in size. They lack the central pallor. In other haematological disorders like IHA, microangiopathic hemolytic anemias the spherocytes show marked anisocytosis not like HS.

MCHC is a sensitive indicator in a case of HS. MCHC > 35-36% is commonly seen in HS. Due to water loss within the cell MCHC will be found elevated. In HS the MCV will be low owing to water loss as well as the red cell membrane.

The most sensitive test for HS is the incubated osmotic fragility test, which is performed after incubating RBCs for 18-24 hours under sterile conditions at 37°C. Osmotic fragility testing with RBCs that have not been incubated may demonstrate haemolysis of HS cells in some patients but is not reliable. This is especially true of newborns, as fetal red blood cells generally are resistant to osmotic haemolysis.

Further studies include to evaluate the specific membrane cytoskeletal abnormalities are available in selected research centres. Several other tests are needed in patients who have experienced an aplastic crisis. Testing for HSV, parvovirus B19, and EBV may help to identify a viral cause for the aplastic crisis. In geriatric age group patients must have an evaluation of their iron status, especially in setting of repeated blood transfusions or if they have received prolonged oral iron supplementation for anaemia. This evaluation includes measurement of serum iron levels and serum ferritin levels. Patients with severe iron overload may present with hepatic dysfunction or cardiovascular problems or diabetes.

If the patient has suspected underlying haemolytic process along with right hypochondriac pain, elevated body temperature, and leukocytosis, imaging studies should be performed to rule out cholelithiasis due to a primary haemolytic disorder.

If there is suspicion of aplastic crisis, further evaluation of white blood cells and thrombocytes should be done. A bone marrow aspiration study and biopsy will aid further to make a correct diagnosis of whether aplasia or megaloblastosis. Risk of kernicterus is increased in newborn babies with ↑ bilirubin caused by HS.

In childrens splenectomy is done for HS after the age of 6 years. It is indicated in children with moderate and severe HS. They will be presenting with severe anaemia and growth retardation. Partial splenectomy is practiced nowadays which will preserve the immune function and the marked reduction in the percentage of haemolysis. [56,57]

**Diagnostic difficulties of Thalasaemias**

A peripheral blood with target cells, and other features of anaemia which may be mild to severe. The MCV will be typically low and the red cell count will be low or normal. RDW will be low in contrast to iron deficiency anaemia. Heinz bodies which represent inclusions with in RBCs consisting of denatured haemoglobin may be seen in PBF. [57] These findings along with other parameters like bilirubin, elevated reticulocyte count, decreased osmotic fragility test, negative coomb's test, hemosiderinuria or hemoglobinuria and reticulocytosis will direct further confirmatory tests in the line of thalassaemias.

Haemoglobin electrophoresis*:* This characteristically shows elevated HbF (10–98%). HbA2 may be normal or increased. In homozygous β0 thalassaemia,HbA is completely lacking, while in β0/β+ and β+/β+ thalassaemias, some amount of HbA is present Haemoglobin electrophoresis with an elevated HbA2 moiety confirms a beta thalasssaemia trait. In case of Sickle-cell β0 thalassaemia HbS is the predominant haemoglobin (70–80%), HbA is absent, and HbA2 (3–5%), and HbF (10–20%) are elevated. Diagnosis is confirmed by demonstrating that one parent has sickle-cell trait and the other has β thalassaemia trait. A characteristic laboratory feature of β thalassaemia minor is elevation of HbA2 (3.5–7.0%). HbF may be mildly increased.

In Haemoglobin Bart's Hydrops Foetalis Syndrome the blood film shows severe anisopoikilocytosis, microcytosis, and erythroblastosis. Haemoglobin level is 5 to 8 g/dl. Large amount of Hb Bart's (approximately 80%) is present in cord blood. Both HbA and HbF are absent since no α chains are synthesised. Globin chain

synthesis studies demonstrate complete absence of α chain synthesis (α/β ratio of 0). Both the parents are obligatory carriers of α0 thalassaemia.

In Haemoglobin H Disease patients have anaemia (haemoglobin 7–10 g/dl), icterus, and hepatosplenomegaly. Transfusions are usually not needed. Blood film shows anisopoikilocytosis, hypochromia, microcytes, and target cells. When blood is incubated with an oxidising dye such as brilliant cresyl blue, inclusion bodies are formed due to precipitation of HbH. If spleen has been removed, preformed HbH inclusions can be shown with methyl violet. Cord blood of the newborns shows 10 to 40% of Hb Bart's that is slowly replaced by HbH during infancy. In adults, HbH ranges between 5 and 40%. Both Hb Bart's and HbH are fast-migrating haemoglobins at alkaline pH (i.e. they move more rapidly than HbA). Globin chain synthesis studies show α/β ratio of 0.2 to 0.4. Family study shows that one parent has α0 thalassaemia trait (- -/αα), and the other has α+ thalassaemia trait (-α/αα), The increased Hb A2 level also is not seen in those with the rare delta-beta thalassemia trait. Increased Hb F level is not specific to beta thalassemia trait.

**SICKLE CELL ANEMIA**

Patients with sickle cell disease will have moderate to severe anaemia, reduced PCV,elevated total count with neutrophilia, low platelet count, decreased ESR and a variable reticulocyte count. Peripheral blood film will show elongated hyper-chromatic sickled cells with other features of anaemia. Howell-jolly bodies will be seen in patient who underwent spleenectomy and hemoglobin solublity test will be positive. But it will not differentiate between a sickle cell anaemia or a trait.

Hemoglobin electrophoresis is the investigation choice for HBS. It differentiation between homozygous and heterozygous variants. The following table describes the variants of sickle cell disease and genotypes, haemoglobin solubility tests results and electrophoretic abnormality.

Table 2.sicle cell disease variants and the HB electrophoresis findindings

| **Sickle cell disease** | **Genotype** | **Clinical manifestations** | **Solubility test** | **Hb electrophoresis** |
|---|---|---|---|---|
| Sickle cell anaemia | βS/βS | Moderate to severe anaemia; crises | + | HbS predominant; no HbA; HbA2 normal |
| Sickle cell β0 thalassaemia | βS/β0 | Moderate anaemia; splenomegaly persists | + | HbS predominant; no HbA; HbA 2 increased |
| Sickle cell trait | βS/βS | No anaemia | + | HbA>HbS |
| Sickle cell β+ thalassaemia | βS/β+ | Mild anaemia | + | HbS>HbA; HbA2 increased |

Typical abnormalities in patients with SCD are anaemia with Hgb level of 5-9 g/dL ,decreased PCV in the range of 17-29 per cent, leucocytosi in the range of 12,000-20,000 cells/mm $^3$, with a predominance of neutrophils, thrombocytosis, decreased erythrocyte sedimentation rate, increased reticulocyte count which may vary depending on the extent of baseline haemolysis .

Peripheral blood smears demonstrate target cells, elongated cells, and characteristic sickle erythrocytes and occurrence of RBCs with Howell-Jolly bodies

indicates that the patient is asplenic along with results positive hemoglobin solubility testing but do not distinguish between sickle cell disease and sickle cell trait.

Hemoglobin electrophoresis helps to identify the individuals who are homozygous for HbS from those who are heterozygous. It also confirms the diagnosis of SCD by demonstrating a single band of HbS (in HbSS) or HbS with another mutant hemoglobin in compound heterozygotes.

Neonatal Screening for Sickle-cell Anaemia

Screening can be carried out to identify those newborns who will later develop sicklecell anaemia. The rationale behind this approach is that preventive measures can be taken to avert serious complications and reduce morbidity and mortality in later life. Screening of newborn can be carried out in communities with increased frequency of sickle-cell gene. This approach is used in USA in African Americans.

In newborns, solubility test and sodium metabisulphite test cannot be used for screening since concentration of HbS is very small (< 10%). Widely used test for this purpose is citrate agar gel electrophoresis at acid pH. Haemolysate from cord blood sample is used. Newborns who will develop sickle-cell anaemia show predominance of HbF, some HbS and absent HbA; those with sickle-cell trait have HbF, HbS, and HbA.

**DIAGNOSIS OF G6PD DEFICIENCY**

During haemolysis, general features of haemolytic anaemia are present. Peripheral blood smear shows: polychromasia, fragmented red cells, spherocytes, bite

cells (red cells having bitten out margins due to plucking out of precipitated haemoglobin by splenic macrophages), and half-ghost cells (one half of red cell appears empty, while other half is filled with haemoglobin). Biochemical investigations reveal unconjugated hyperbilirubinaemia, haemoglobinaemia, haemoglobinuria , and decreased or absent haptoglobin.

*Heinz Bodies*

They can be detected after vital staining with methyl violet. They are usually seen immediately following haemolysis. Heinz bodies are deep purple small inclusions attached to red cell membrane. In addition to G6PD deficiency, they are also seen in unstable haemoglobin disease. Qualitative or Screening tests like Fluorescent spot test, Methaemoglobin reduction test,and Dye decolourisation tests are used to screen this disease. During acute haemolytic episode, test for G6PD deficiency may yield negative result due to reticulocytosis (since reticulocytes have high G6PD content). In suspected cases, the test should be repeated about 6 weeks after the haemolytic episode.

***Quantitative assay of G6PD***

This test is available only in reference laboratories. Haemolysate is incubated with glucose-6-phosphate. The rate of reduction of NADP to NADPH depends upon G6PD activity in the lysate. The rate of production of NADPH is measured in a spectrophotometer at 340 nm and G6PD activity is derived. Genetic testing consists of DNA-based genotyping and sequencing, which helps identify hundreds of mutations associated with G6PD deficiency, including many region-specific common variants.

The molecular analysis may be useful for population screening, family studies, females, and prenatal diagnosis.

## MICROANGIOPATHIC HAEMOLYTIC ANAEMIA

This refers to haemolytic anaemia resulting from intravascular fragmentation and lysis of red cells due to alteration in small blood vessels. Usually direct damage to red cells occurs when they pass through the fibrin strands deposited in the microcirculation. Common causes of microangiopathic haemolytic anaemia are Thrombotic thrombocytopaenic purpura, HUS DIC, Malignant hypertension, disseminated malignancy Several infections and generalised vasculitis due to immunologic diseases. The presentation depends on the underlying disease and the anemia is of variable severity. The characteristic feature on peripheral blood smear is occurrence of fragmented red cells or schistocytes**.** Schistocytes include small red cell fragments with 1 to 3 sharp spicules as well as large helmet-shaped red cells from which fragments have been split off. Evidence of intravascular haemolysis and consumptive coagulopathy is often present.

Consistent findings in HUS include anemia and thrombocytopenia, with fragmented red blood cells. WBC differential may reveal a left shift (ie, immature WBCs, including bands, myelocytes, metamyelocytes). Individuals with Shiga toxin–producing *E coli* hemolytic-uremic syndrome (STEC-HUS) may have very high WBC levels, in the range of 50,000-60,000/µL. Antiglobin tests are negative, except with *S.*pneumoniae –associated hemolytic-uremic syndrome. Reticulocyte count is increased and levels of serum haptoglobin, are decreased. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) are normal with the levels of fibrin degradation products being increased.

Testing for ADAMTS13 activity may help differentiate between atypical hemolytic-uremic syndrome (aHUS) and thrombotic thrombocytopenic purpura (TTP). ADAMTS13 activity less than 10% is consistent with a diagnosis of TTP. Patients with decreased levels of ADAMTS13 activity should also be tested for the presence of antibody to ADAMTS13. If plasma exchange is planned, specimens should be obtained before starting plasma exchange, as the donor plasma may confound the results. Rapid diagnosis is very helpful. Several laboratories offer 24-hour turnaround service.

**Utility of an isolated screening panel**

Hemolysis is due a wide variety of etiology. Some of the diseases are acute like immune haemolytic anemias and some are chronic like the thalasaemias. Some haemolytic diseases are associated with autoimmune diseases which will be diagnosed with other manifestations of the primary disease. but when the compensatory machanisms are acting in balance there will not be a clinical manifestation of disease. So when the process of hemolysis is not compensated by bone marrow erythropoiesis. It manifests as any other case of anemia. Whenever a clinical suspicion of haemolytic anaemia is made in our scenario patient will be .a n isolated panel is necessary for

1. Establishing the haemolytic process
2. compensatory state of the body
3. To establish etiology
4. Guiding for further investigations

5. To avoid unnecessary tests

Establishing the ongoing hemolytic process can be done by doing a CBC, serum unconjugated bilurubin,and a reticulocyte count. Red cell kinetic and survival studies are the definitive way of establishing haemolytic anemia. The severity of anemia and the reiculocyte count infers the severity of the disease and the ability of the body to compensate for the loss. The peripheral smear combined with CBC guides in the direction of further work up. By carful study of peripheral smear the following inferences can be arrived. spherocytes ,elliptocytes, pyropoikilocytes can be made out at the peripheral smear. Low MCV and with microcytic hypochromic anemia will point towards thalasaemias. Normal MCV, target cells, poikilocytes like sickle cells, and Hb crystals infer hemoglobinopathies. Schistocyes are seen in microangiopathic hemolysis. Fever with unusual red cell inclusions point towards infections like malaria in Indian scenario. Agglutination is seen in immune haemolytic anemias which can be furthur evaluated by doing a cooomb's test. Bite cell may be seen in membranopahies.

The extra vascular hemolysis is established by doing a simple test i.e hemosiderinuria. It establishes a chronic haemolytic process. The intravaswcular hemolysis can be established by hemoglobiuria. Even though osmotic fragility test is not confirmatory for a case of hereditary Spherocytosis it acts as a clue to further investigations like family studies,or flowctometry.

Unlike the developed world where health related expenses are borne by insurance,in our country The patient has to spent for most of the tests. So unnecessary tests will increase the cost of treatment and in some cases patients abandons treatment as it is

expensive. So a simple isolated screening panel will be of immense value in our system. The panel of tests are CBC, peripheral smear, reiculocyte count, unconjugated bilurubin, coomb's test,osmotic fragility test, benzidine test for hemoglobinuria and perls test for hemosiderinuria whether serve the purpose wil be analyed in this study.

**Role of each tests**

**CBC (COMPLETE BLOOD COUNT)**

Complete blood count is an important tool in diagnostic work up for anemias. Complete blood count incorporates total WBC count, RBC count, differential count (DC), haemoglobin concentration (Hb), mean corpuscular volume(MCV),packed cell volume (PCV),mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width(RDW). The indices pertaining to Red blood cells are called RBC indices. By analysing values and histographs various types of anemias like microcytic hypochromic anemias, megaloblastic anemias, various haemolytic anemias and haematological malignancies, can be diagnosed. In the following paragagrahs the parameters that vital to the diagnosis of hemolytic anemia will be analysed in brief.

**TOTAL COUNT**

The toal count signifies the total WBC count per cubic millimeter. Whenever there is inappropriate rise in total count a careful examination of peripheral smear to be done yo rule out haematological malignancies or normoblastemia (nRBCs). Nucleated RBCs (nRBCs) not seen usually in peripheral smear beyond neonatal period. Their appearance beyond 5 th day of life in a normal infant signifies some

serious underlying etiology .premature birth, fetal hypoxia ,can increase the number. But beyond the new born period the presence of nRBCs is associated with malignant neoplasms,disorders of bone marrow, and other serious disorders. normoblasts are less deformable and and rarely enter circulation. whenever the wbc count grossly elevated as given by the automated hematology analysers ,the peripheral smear of the patient to carefully reviewed for blasts or nRBCs.

Although studies show that even one nRBC in the peripheral blood of adults may indicate a serious disease, clinicians and laboratory professionals do not agree on its clinical significance, when they are not supported by other data. In primary health care units, normoblastemia is rare and may signify a pathologic condition. In contrast, normoblastemia is a common finding in an acute care hospital. As a result, NRBCs may be perceived as ordinary cells of questionable clinical significance; in these cases, the importance of normoblastemia is relative and depends on the type of hospital and patient population. [100] The corrected WBC can be calculated from this formula.

**Corrected WBC count=100 x WBC÷100+nRBC**

In advanced hematology analysers the corrected WBC count can be obtained.

**Red cell indices**

Red cell indices are derived from the values of red cell count, haemoglobin (Hb) concentration, and packed cell volume (PCV). Red cell indices obtained by manual methods are often inaccurate. Electronic haematology cell analysers more reliably perform them.

The normal ranges of red cell indices in adults are as follows:

MCV = 80–100 fl

MCH = 27–32 pg

MCHC= 32–36 g/dl

**1. Mean corpuscular volume (MCV):** MCV represents the average volume of a single red cell. It is expressed as femtolitres or fl (1 fl = 10-15 litres). MCV is calculated manually as follows:

$$\text{Mean cell volume (MCV) in femtolitres} = \frac{\text{Packed cell volume in\%}}{\text{Red cell count in million per cmm}} \times 10$$

Anaemias are classified as normocytic, microcytic, and macrocytic on the basis of MCV values. Since MCV measures average cell volume, it may be normal even though there is marked variation in size of red cells (anisocytosis). Normal range of MCV is (80-100 fl). Low MCV is seen in thalassaemia minor,iron deficiency anemia,sideroblastic anemia and anemia of chronic disease. High MCV is seen in Vit B12 deficiency,bone marrow failure,and alcoholism.

**2. Mean corpuscular haemoglobin (MCH):**

This is the average amount of haemoglobin in each red cell. It is expressed in picograms or pg (1 pg = 10-12 of a gram) and is derived manually from the following formula:

$$\text{MCH (in picograms (pg))} = \frac{\text{Haemoglobin (g/dl)} \times 10}{\text{Red cell count in million/cmm.}}$$

Low MCH is found in microcytic hypochromic anaemia, while high MCH in macrocytic anaemia. Normal range of MCHC is 27-32 pg.

**3. Mean corpuscular haemoglobin concentration (MCHC):** This represents the average concentration of haemoglobin in a given volume of packed red cells. It is expressed in grams/dl and calculated as follows:

$$\text{MCHC in g/dl 100} = \frac{\text{Hb (g/dl)}}{\text{PCV (\%)}} \times 100$$

Normal range of MCHC is 32- 36 g/dl. Low MCHC haemoglobin concentration (MCHC) in g/dl 100 occurs in microcytic hypochromic anaemia. An increase in MCHC occurs in hereditary spherocytosis. Increased MCHC is is seen in HS,sickle cell anemia,B12 & folate deficiencyHbC (homozygous) and low MCHC is seen in Iron deficiency anaemia and haemorrhagic conditions.

**Red cell distribution width (RDW):** Automated analysers produce a quantitative measurement of the variation in cell volume, an equivalent of the microscopic assessment of the degree of anisocytosis. This parameter has been named the ‘red cell distribution width’.

The RDW is derived from pulse height analysis and can be expressed either as the standard deviation (SD) in fl or as the coefficient of variation (CV) (%) of the measurements of the red cell volume. The RDW SD is measured by calculating

the width in fl at the 20% height level of the red cell size distribution histogram and the RDW CV is calculated mathematically as the coefficient of variation,

i.e. RDW (CV)= 1SD/MCV X 100%.

Most instruments express the RDW as the SD, but Sysmex instruments and the Beckman Coulter instrument express it as both SD and CV.

The normal reference range is in the order of 12.8 +/- 1.2% as CV and FOR SD 42.5 +/-_ 3.5 fl as SD.

However, widely different ranges have been reported; therefore it is important for laboratories to determine their own reference ranges. The RDW expressed as the CV has been found of some value in distinguishing between iron deficiency (RDW usually increased) and thalassaemia trait (RDW usually normal) and between megaloblastic anaemia (RDW often increased) and other causes of macrocytosis (RDW more often normal).

The following flow chart can be of useful interpretation of various haemolytic anemias.

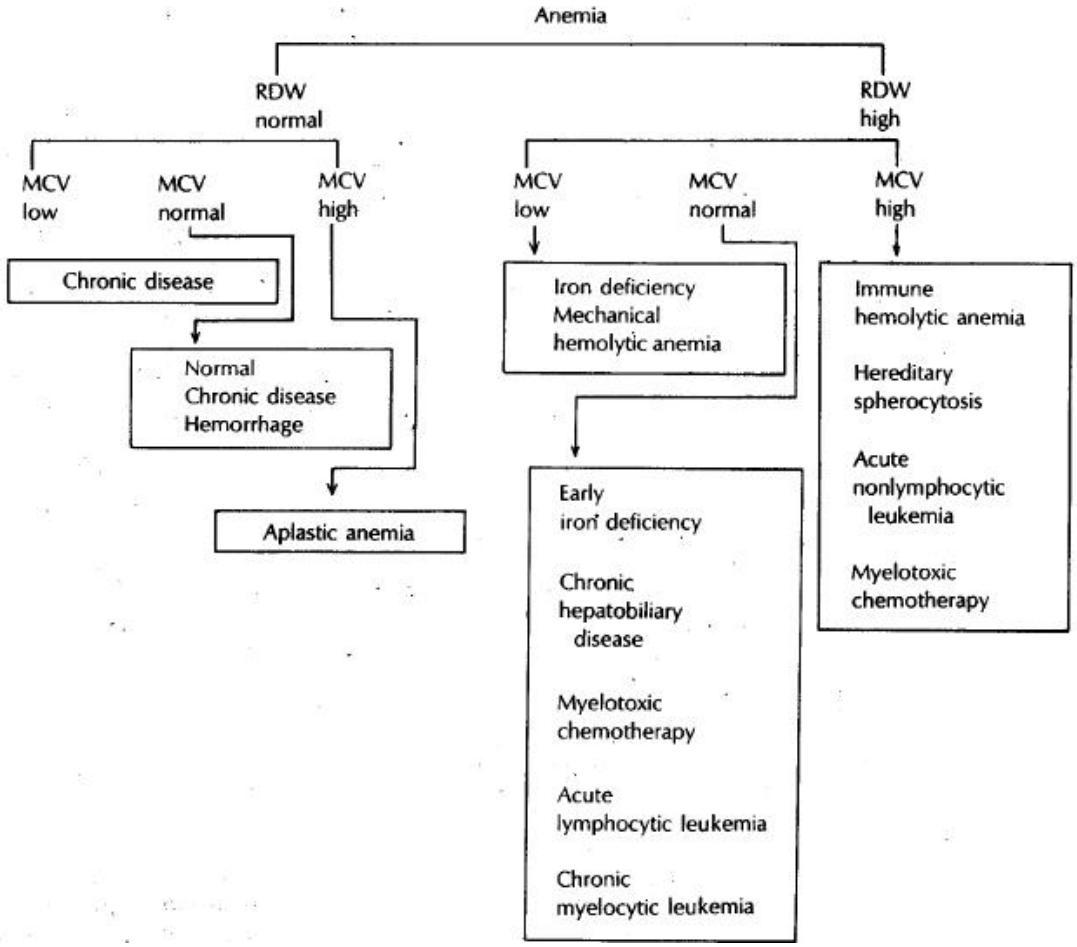



**Figure – 3. Correlation of RDW with other red cell indices**

**Peripheral smear**

Morphology of various cell lineages can be analyed with the help of peripheral smear. PBF is a vital tool to a hematopathologist. Progression of a haematological disorder and its response to treatment can be assessed by a peripheral smear. Literature reveals more than seventy percent of clinical decisions and diagnoses are supported by a good clinical haematologist. [59]. The PBF exposes the morphology of peripheral blood cells, which ensures its place in the morphologic diagnosis of various

primary and secondary blood and blood related diseases such as haemolytic anemias and haematological malignancies.

Romanosky stains are commonly employed in staining of PBF which gives a differential staining in a way that it stains the acidic and basic components in a different colours.[60] Leishman stain is most commonly used stain in our laboratory which is composed of eosin (acidic component) and polychrome methylene blue (basic component). Some laboratories use MGG (May-Grunwald Giemsa) or Wright-Giemsa stain. [61]

Interpreting a peripheral blood film is done by preferably by a hematopathologist especially for slides that show pathology.[62,63] The method of viewing a slide is to start from the tail of the smear and concentrate on the junction of the body and the tail where the cells start just overlapping. The head portion is significant while examining the slide for hemoparasites. The feathered portion should be examined when a haematological malignancy is suspected and platelet clumps are mostly concentrated near the tail of the smear. White reporting a smear one should take into account, the morphology, inclusions of all cell lineage. However, fragmented cells and artifacts also should be taken into account.

The shape of normal RBC is biconcave disc-shaped with 7–8 µm. Each cell has central palor occupying about 1/3$^{rd}$ of total volume, surrounded by pink colour imparted by Romanowsky dye. The eosin imparts the pink colour which is taken up by the cytoplasm of red blood cells which is acidic [64]. Red cells show wide variation in size which is designated as anisocytosis and it can be either microcytic macrocytic,

macro-ovalocytic or normocytic. A mean-corpuscular volume of < 74fl suggests microcytosis while if it is more than 96% fl suggests macrocytosis. Ovalo-cytosis is seen in megaloblastic anaemias (B6 or B12 deficiency), drug-induced or mielodysplastic syndrome. In liver disease and alcoholism there will be plenty of round macrocytes in PBF.

Table no.3 showing various peripheral smear findings and their differential diagnosis

| **Red Cell Shapes** | **Differential Diagnosis** |
|---|---|
| Irreversibly sickled red cells (Drepanocytes) | Sickle cell syndromes (SS, SC, Sβthalassemia) |
| Target cells (codocytes, Mexican hat cells)<br><br>(Fig 7) | Sickle cell disease, haemoglobin C trait, haemoglobin CC disease, thalassemia, iron deficienty, Liver disease (Cholestasis) asplenia |
| Fragmented red cells (schistocytes, helmet cells, keratocytes) | Thrombotic micro-agniopathic haemolytic anaemias such as Disseminated intravascular coagulopathy, (DIC), thrombotic thrombocytopenic purpura, haemolytic uraemic syndrome |
| Burr cells (echinocytes, crenated red cells) | In-vitro artefact following prolonged storage or slow drying of the smear due to high humidity, uramia, Malnutrition |
| Spur cells (acanthocytes) | Liver disease, Renal failure, Abetalipoproteinaemia, Spur cell anaemia, pyruvate kinase deficiency |
| Tear drop cells (dacrocytes) | Myelofibrosis, Myelophthsia (marrow infiltrations), Extramedullary haemopoiesis, Hereditary elliptocytosis, Hereditary pyropoikilocytosis, Severe iron deficiency, Megaloblastic anaemia, Thalassemias, Myelodysplastic syndrome |
| Bite cells (degmacytes) | G6PD deficiency, Oxidative stress, unstable haemoglobins, congenital heniz body anaemia |
| Pencil cells | Iron deficiency |
| Stomatocytes | Artifact (due to slow drying in humid environment), Liver disease, alcoholism, Obstructive lung disease |

| | |
|---|---|
| Elliptocytes(fig7) | Hereditary Elliptocytosis (>25%) |
| Basket cells (half ghost cells Blister cells) spherocytes | Oxidant damage, G6PD deficiency, Unstable haemoglobins Hereditary spherocytosis, ABO incompatibility, Autoimmune haemolytic anamia (warm antibody type) Severe burns |

Red cell inclusions are due to defective maturation of RBC. oxidant injury to the cells or infections. In post-splenectomy individuals and in anatomical and functional asplenism, Howell jolly bodies are seen which are remnants of DNA. Some of the hemoglobinopathies, some of the poisoning like led are arsenic and in some severe infection, Basophilic stipplings which are denatured RNA remnants found in the PBF. Reticulocytes exhibits polychromasia on Rowmanosky stained slides. They are released in premature from the marrow sinusoids and taken about 1 – 2 days for maturation. Increased nucleated RBCs in PBF indicates severe stress on marrow causing premature release of RBCs. When, premature RBCs along with precursors of WBC lineage like band forms is termed as leucoerythroblastic picture.

A haemoglobin content is directly proportional to the colour of the red cells. Hyperchromia signifies increased haemoglobin content, whereas hypochromia indicates decreased haemoglobin content. Hyperchromatic RBCs lacks the palar central area which can be larger (Polychromotophilic RBCs) or small cells (microspherocytes) Pyknocytes are irregularly contracted cells which have and abnormal cells with hyperchromia. Uniform sized spherocytes are seen in hereditary spherocytosis (HS) where as microspherocytes are seen in IHA (immune haemolytic anaemia) and burns. Crenated red cells are due to storage for long hours. Leptocytes which ae hypochromatic large cells with the thin rim are seen in liver diseases.

The WBC (white blood cell) morphology and much of WBC disorders can be diagnosed with peripheral smear. The same applies platelets as well. Wide variations in WBC morphology and count can be seen in a variety of haemolytic disorders. Whenever there is WBC more than 5 per hpf, it is designated as leucocytosis, whereas WBC < 2 signifies lucopenia. The accuracy will be better, if more number of fields are examined. So, cell count estimation of leucocytes will be better in a low-power. Especially, when conditions like pancytopenia bone-marrow aplasia are suspected.

PBF is of diagnostic significance in certain conditions like sickle cell disease, hereditary eliptocytosis or spherocytosis. So, an accurate diagnosis warrants a sound clinical knowledge of different cytomorphological abnormalities of a disease with good laboratory experience.

**RETICULOCYTE COUNT**

Reticulocytes are anucleated cells, more rounded in shape and 20 per cent greater, in volume, than the erythrocytes. They produce polychromatic slides, when stained with panoptic dyes (Romanowski), as the result of mature red blood cells with hemoglobin, synthesized during maturation, and also reticulocytes with ribonucleic acid residues. Microscopic studies reveals characteristic aspect of reticulum, when the residues are stained with new methylene blue or brilliant cresyl blue dyes.[65]

Based on the granules within the reticulocytes can be graded in to 4 groups. Group 1 is the most immature group which will have more RNA contents. Group-2 & 3 are intermediate groups which will have RNA content in between group 1 and 4. Group 1 will have lesser RNA content which will appear as dots within the RBCs. So, reticulocyte count is a common to for assessing marrow activity with respect to

ongoing haemolytic process. It is also useful after bone-marrow transplant and post-chemotherapy assessment of marrow activity. The reticulocyte counting by manual method was first explained during 1930, and still practised world-wide. Compared to costlier automated method, the manual one is cheaper. However, manual counting presents some factors that cause great variation.

Peebles *et al*[68] reported inter observer variation coefficients between 25 per cent and 50 per cent, considered higher than the expected one. For more precise results, manual counting must follow standardized criteria of execution and identification. As suggested by the H-44 technique norms of the National Committee of Clinical Laboratory Standards (NCCLS) and of the International Committee for Standardization in Hematology (ICSH), some criteria must be observed for a good performance of reticulocyte manual counting with special attention to the differentiation among reticulocyte granules, Pappenheimer, Heinz, and Howell-Jolly bodies, or remaining dyes and taking special care in the preparation of the reticulocyte slides avoiding overlapping of cells and also accounting the number of evaluated cells. [69]

In rural regions of India, manual technique is widely practised. By staining with new methylene blue or brilliant cresyl blue and interpreted by a trained microscopist or hematopathologist. It is simple but it has its own flaws, there is a 25 – 50 percent inter-observer variation .[70,71] There are many factors that affects the results of the manual method like experience of the hematopathologist usage of ocular inset

and the quality of the smear and the total number of fields counted. [72] However, the accuracy and precision can be obtained in automated machines.[76]

Non-fluorescent (Oxazine 750, new methylene blue), and fluorescent (thiazone orange, auramin O, cyanene) dyes are used by the principle of flowcytometry to estimate the reticulocytes. [73]Introducing maturation indices and volume measurements, automated reticulocyte counting adds a vital method for analysis. [74-76] The proper evaluation of reticulocyte count is hampered by the presence of cells like schistocytes nRBCs, cell debris, hemoparasites and giant platelets.

The peripheral blood sample is stained with supravital stain (brilliant cresyl blue or New Methylene blue). These are basic dyes that have the ability to react with ribosome and nucleic acids of reticulocytes while it still alive. The neuclic acid dye reaction form a blue precipitate of granules or filaments.

The normal range of values for reticulocytes in the blood depends on the clinical condition and the laboratory standards, but the value of 0.5 per cent to 1.5 per cent is considered normal. However, in case of anemia the reticulocyte count should high. However, in many times the proportionate elevation cannot be established. So, the reticulocyte production index is an important parameter calculated from retic count and the RBC count which will be used to assess the appropriateness of the retic count with regarding to the given clinical scenario.

The reticulocyte count is also useful in the management of patient therapy. It can be used to follow idiopathic myelofibrosis, [79] evaluate a patient's responsiveness to folate, iron, or B 12 supplementation, follow the progress of bone marrow

transplant,[80] and assess the impact of chemotherapy and radiation on hematopoieticfunction.[81]

**Bilirubin**

Bilirubin is an endogenous compound and is yellow in colour. that can be toxic when in excess .Bilirubin has a yellow colour with, for the unconjugated molecule a typical spectrographical peak at 450 nm [82]. Bilirubin plays a protective role when is mildly elevated. It protects against neoplasms and CVS diseases. Bilirubin is produced by the breakdown RBC at the reticuloendothelial system. It is derived from the haem moiety of haemoglobin.the daily production of about 200-300 mg in an adult [83]per 24 hours. More bilurubin is produced in neonates compared to adults.conjugation of bilirubin occurs in the hepatic microsomes. The conjugated bilurubin excreted in bile which is mediated by a transporter called MRP2 which belong to the ATP .[85] the bilurubin is transported to intestine where major part of it undergoes enzymatic breakdown. A minor portion of it gets deconjugated and reabsorbed and will remain as UCB. Disorders of Bilurubin metabolism can be categorised under unconjugated bilirubinemia,conjugated bilirubinemia,and mixed type.it is based on the DB/TB ratio.if it is unconjugated bilirubinemia the value willbe 20-30%,>70% signifies conjugated bilirubinemia and the values will be between these two in mixed type. [86]In most of the laboratories esmation of TB and CB will be done and UCB is calculated from the above values. In haemolysis the bilurubin content will be abnormally high.[87]whenever there is haemolysis the bone marrow will compensate with production of more immature cells pushed into circulation to compensate the loss. These immature cells i.e normoblasts which have a short half life

compared to the matured ones .so they undergo earlier lysis.thus increasing the UCB percentage.

Haemolysis is one of the most common causes of unconjugated hyperbilirubinaemia. [88] During activated synthesis of RBC in the bone marrow compartment due to any etiology. Anemia may not be evident but unconjugated bilirubinemiacan be evident due to accelerated destruction of RBC. Elevated bilirubin levels are seen in haemolytic diseases like thalassaemia, sickle cell anemia,and hereditary spherocytosis . it can also be markedly elevated in diseases like Gilbert syndrome.[89,90]

**Principle**

The most widely followed and simple method is Diazo method of Pearman &lee(end point).the principle is that bilurubin reacts with diazotised sulphanilic acid in acidic medium to form pink coloured azobilurubin with absorbance that is directly proportional to bilurubin concentration .direct bilurubin, being water soluble directly reacts in acidic medium. However indirect or unconjugated bilurubin is solubulised using a surfactant and then it reacts similar to direct bilurubin.

**COOMB'S TEST**

Anti human globulin test (AHG) comprising of both Direct Coombs' Test (DAT) and Indirect Coombs' Test (IAT) can be used to detect RBC sensitized with Ig G auto and alloantibodies, complement occurring in vivo and vitro. Anti human globulin test is the mainstay of any immune hematological work up. Interpretation of AHG is a significant step and which should be mandatory for the better management of patients.

Coombs' test also known as Anti Human Globulin test (AHG) was described in 1945, by Coombs and associates for detection of weak and nonagglutinating Rh antibodies in serum. Direct antiglobulin test (DAT) was described by Coombs and coauthors in 1946 describing use of AHG to detect in vivo sensitization of RBC of babies suffering from hemolytic disease of new born (HDN).[91] detect RBC sensitized with Ig G alloantibodies.

The Antiglobulin test can be used to Ig G autoantibodies and complement components occurring invivo or in vitro. Indirect Antiglobulin Test (IAT) refers to the use of AHG in detecting in vitro sensitization of RBC as two-stage technique. The present study deal with the evaluation of DAT and IAT in all the immunohematological cases keeping in view to the past and present medical history and presentations.

DAT, 1-2 drops of EDTA anticoagulated patients' red cells were washed three times with isotonic saline in test tubes. After decanting the supernatant completely following the third wash, to the dry botton Polyspecific AHG was added and incubated in the room temperature for 5 minute. The tubes were centrifuged at 1000rpm for 1 minute. Immediately after gentle re-suspension of the agglutination, macroscopic examination was done using lighted agglutination viewer. When the result was negative, we examined for the agglutination under microscope. To negative result interpreted microscopically, one drop of Ig G-coated Coombs Control Cell was added to the tube and centrifuged lightly for 1 minute at 1,000 rpm. It was immediately re-suspended gently and examined macroscopically for agglutination. A positive reaction at this state confirmed a negative test. If the result was negative after addition of the Ig G

coated Coombs Control Cells, the test was reported to be invalid and was repeated. For IAT,1 to 2 drops of patient's serum 1 drop of 5% saline-suspended reagent group O cells is added in the test tubes and mixed. The test tubes were then centrifuged at 1000 rpm for 1 minute and were observed for hemolysis and agglutination. The tubes were incubated at 37 C for 30 to 60 minutes followed centrifugation and observation was done for hemolysis and agglutination. Again, red cells were washed for three times with saline; completely decanting the final wash.AHG was added to the dry red cell button and was mixed well. The test tubes were centrifuged and were observed for agglutination. Results were graded and were recorded. Validity of the negative results was confirmed by addition of IgG-coated red cells.

Antiglobulin test detects red cell antibodies which are bound and do not produce direct agglutination.AHG reacts with human antibodies and complement bound to red cells and also unbound to cells, free in serum.AHG sera can be used for performing DAT and IAT.DAT demonstrate in-vivo sensitization of red cells and is performed on the patients' washed red cells to which AHG is to be added.IAT demonstrates in-vitro reactions between red cells and antibodies which can be performed on serum of patients from which red cells are washed to remove unbound globulins.2DAT is the investigation of choice in hemolytic disease of the fetus and newborn (HDFN), transfusion reactions, autoimmune hemolytic anemia (AIHA), and drug-induced immune hemolysis. The predictive value of positive DAT is 83% in a patient with hemolytic anemia, and only 1.4% in a patient without hemolytic anemia.[92]

IAT is used for antibody detection, antibody identification, cross matching and blood group phenotyping. DAT is performed by testing freshly washed red cells

directly with Antiglobulin reagents containing anti-IgG and anti-C3d.False negative or weaker result can be obtained if the washed red cells are allowed to sit before testing with anti-IgG or delay in the reading. False positive results can arise in degradation of specimens causing non-specific binding of DAT reagents. Causes of false positivity in Antiglobulin test include over-centrifugation,under agitation, prolonged delay in testing, clotted specimen, reagent issues and spontaneous agglutination.[93,94] Causes of false negative results include neutralization of AHG reagent by failure to wash inadequately, interruption in testing, improper reagent storage,over-centrifugation.[95]Positive DATs are reported in 1:1000 upto 1:14,000 blood donors and 1% to 15% of hospital Patients.[96]

**OSMOTIC FRAGILITY TEST**

The confirmatory test for HS is osmotic fragility (OF) test which determines susceptibility of red cells to haemolysis during osmotic stress conditions. Haemolysis can be measured when the cells are suspended in decreasing concentrations of hypotonic saline solutions where, the water enters in to the red cells. Due to their biconcave shape, normal red cells can withstand hypotonicity by increasing their volume to certain limit and after that they cannot swell leading to cell burst, by discharging their haemoglobin into the supernatant. Spherocytes have a decreased surface to volume ratio and therefore, they are able to withstand less swelling than normal and are osmotically fragile, i.e. haemolysis occurs in more concentrated solution than normal cells. Osmotic fragility test are performed by Highest concentration of saline at which haemolysis starts (normal 0.50 g/dl NaCl) and the highest concentration of saline at which it is complete (normal 0.30 g/dl of NaCl) or

by Concentration of saline showing 50 per cent lysis (Median corpuscular fragility) or by normal value is 0.40 to 0.45 g/dl NaCl. Increased value is proportional fragility. A graph may be plotted with amount of haemolysis on vertical axis and saline concentrations on horizontal axis. Normal osmotic fragility curve is sigmoid shaped. Shift of the curve to the right indicates increased osmotic fragility.

Osmotic Fragility Test after Incubation

Osmotic Fragility Test is performed by incubating sterile blood at 37°C for 24 hours and in hypotonic saline solutions. During incubation, metabolic deprivation of spherocytes occurs mainly due to decreased concentration of glucose, with resultant membrane destabilisation, loss of membrane, and enhancement of spherical shape. Due to the failure of membrane pump with accumulation of sodium and water also counterpart this lysis. The sensitivity of the test is thus increased. Incubated OF test may, however, be normal in 10-20 per cent of patients with HS .

The osmotic fragility is increased in any disorder associated with spherocytosis and is thus not specific for HS. Normal OF test result does not rule out HS as it may be normal in mildly affected patients. Diagnosis of hereditary spherocytosis is usually easily made on the basis of mild to moderate anaemia with spherocytosis, splenomegaly, jaundice, increased osmotic fragility, and evidence of hereditary spherocytosis in the first-degree relative. Sometimes anaemia is mild or absent and the patient may first present with isolated splenomegaly, gallstones, or “aplastic crisis”. Clinical evaluation and examination of blood film for spherocytes are necessary for correct diagnosis.

A spherocyte is a small sized red cell, does not have central pallor and appears densely haemoglobinised (hyperchromic) (fig. 9). Spherocytes, in not only unique to HS and are also found in other disorders such as immune haemolytic anaemias, ABO haemolytic disease of the newborn, haemolytic transfusion reactions, and burns. Increased osmotic fragility is due to spherocytosis, which may result from a variety of causes (HS, immune haemolysis, burns, etc.). Therefore, OF test is not specific for diagnosis of HS and has certain limitations. OF test is normal in patients with mild HS having very few spherocytes in their blood. Incubation at $37^0$ C increasesthe sensitivity of OFT. This test is time-consuming and a tedious one and if spherocytes are present on blood smear, adds little to diagnosis. Decreased osmotic fragility (increased resistance to lysis in hypotonic solutions) is seen in iron deficiency anaemia, thalassaemia, sickle-cell disease, and liver disease and hence result negative for patients with above said disorders. OF test cannot distinguish between causes of spherocytosis.

**Hemoglobinuria**

Red blood cells contains haemoglobin, iron rich molecule helps transport $O_2$ and $CO_2$ through the body. RBC have life span approximately of 120 days and they undergo lysis. This occurs in the spleen, bone marrow, and liver. If there is intravascular hemolysis RBCs break down with in the blood vessels, the fragmented cells move freely in the bloodstream. Higher concentration of Hb begins to appears in the urine and such condition is termed as haemoglobinuria leading to intravascular hemolysis.

Diagnostic symptoms *viz.,* Acute haemolytic conditions like incompatible blood transfusion Hemolytic anemia due to drugs and chemicals, Favism, Paroxysmal cold hemoglobunuria, March (exertional) hemoglobunuria, Haemolytic anemia associated with eclampsia, Haemolytic uraemic syndrome, Haemolytic anemia due to burns ,Snake and spider bites. Chronic causes are PNH, Cardiac hemolytic anemia and Cold haemagglutination disease are some of the auses of hemoglobinuria include

Occurrences of Hemoglobinuria may be evidently clear from random urine sampling or it can be detected in urine samples obtained in successive intervals. Hemoglobinuria may vary dramatically with in a same day or even hour to hour. Haemoglobinuria is excretion of free haemoglobin in urine, develops when plasma haptoglobin cannot bind any more haemoglobin.

The reabsorbed haemoglobin stored as ferritin or haemosiderin in proximal renal tubular epithelial cells. And as the result their shedding causes, haemosiderinuria in urine. The confirmation test for Haemoglobinuria is benzidine or orthotoluidine test on urine sample and urinary sediment can be identified by Prussian blue staining.

This is actually an oxidation reaction, where the peroxidase activity of hemoglobin in urine decomposes hydrogen peroxide and liberates oxygen, which oxidizes benzidine to form a green- blue colored complex and this forms the confirmative test for hemoglobinuria, myoglobinuria and hematuria. This test should be avoided during menstruation cycle because false positive results will be seen in women during menstruation, due to contamination of urine with menstrual blood. Hematuria can be differentiated from hemoglobunuria by microscopical examination alone. Even positive results for occult blood tests, RBC cannot be found in such hemoglobunuria conditions.

## Hemosiderinuria

Hemosiderinuria (syn. haemosiderinuria), defined as "brown urine", occurs in addition with chronic intravascular hemolysis. Hemosiderin is nothing but a form of iron that results from the denaturation of ferritin. These insoluble granules are identified by staining with Prussian blue color which grows in size that can be observed microscopically in the urine sediment. Hemosiderin granules are found in the urine sediment 2 to 3 days after a severe hemolytic episode (e.g., transfusion reaction, paroxysmal nocturnal hemoglobinuria). the plasma Hb level is between 50 to 200 mg/dL following hemolysis. if the haptoglobin is incapable of binding Hb, and immediately passes through the renal glomerular system. Part of the Hb is absorbed by the proximal tubules where the hemoglobin is converted to hemosiderin. As a result these tubular cells shed into the urine, as hemosiderinuria. The unabsorbed forms in tubular cells end into hemoglobinuria. Hemosiderin is seen as yellow-brown granules that are free or in epithelial cells and occasionally in casts in acidic or neutral urine.

## Rous Method

Prussian blue stained Hemosiderin can be identified as free floating or in epithelial which was first studied by Rous in 1918 to identify urinary siderosis, where the granules appear as characteristic blue.[97]Hence this reaction gets its name, the Prussian blue reaction, which is also called as the *Rous test.* For this test *c*oncentrated urine sediment is used for examinination and for the presence of coarse yellow-brown hemosiderin granules, free floating or within casts or tubular epithelial cells is positive identification. The urine sediment is suspended in a freshly prepared solution of

potassium ferricyanide–HCl and is allowed to stand at room temperature for 10 minutes and centrifuged and the sediment is used reexamined for the presence of coarse blue granules. The hemosiderin iron causes the granules to stain Prussian blue. Sometimes this reaction may be delayed so for further confirmation, the negative sediments are examined after 30 minutes.[98]

A smear is prepared with sendiments of centrifuged 10 ml of early morning sample and which is fixed by methanol. Later stained using mixure of 2 per cent potassium ferrocyanide and 1 per cent HCl in equal volumes and kept undisturbed for 10-15 minutes. Further a counter stained, eosin dark blue granules were used and observed under microscope as in bone morrow indicates a positive test.

**Materials and methods**

This chapter deals with the methodology and procedures for the prospective study on types haemolytic anaemia. The present study was carried out at the Department of Pathology at Tirunelveli Medical College and Hospital during September 2013 to September 2015. The study population are irrespective of their age and sex the patients diagnosed clinically as haemolytic anaemia and the patients referred to clinical pathology lab with anaemia and peripheral smear study showing features of haemolysis.

**Inclusion criteria**

This study comprises all catogories of patients irrespective of the age and sex who possess the evidence of haemolysis and haemolysis of both intravascular and extravascular etiology.

**Exclusion criteria**

There are some lacuna that has to be omitted is this due course of research which comprises, Anaemia caused by nutritional cause, hematopoietic malignancy and aplasias. Evidence of clinical disease but without laboratory evidence is not included in the study. The patients who possess the necessitate symptoms were examined and their absolute details were acquired from them in person and were documented and filed. Complete clinical history of the patients presenting with symptoms *viz.*, pallor, dyspnoea, hematuria and fatigue and their order of presentation, onset and duration of symptoms, family history, history of co morbid conditions like diabetes, hypertension, autoimmune diseases and prosthetic valve were obtained in detail and the acquired information were addition support for this research.

A complete clinical examination was performed as per the diagnostic procedure. Patient is specifically examined for spleenomegaly and jaundice. The blood sample and urine samples of selected patients were collected and labelled for further analysis and investigated.

**Collection of blood:**

From each patient undergoing evaluation of hemolysis 6 ml of venous blood were collected by venipuncture at the ante cubital fossa under aseptic precautions and were labelled for further diagnostic exploration. Nearly 6 ml of blood is separated into three different tubes with 2ml each. First one in the EDTA tube is used for complete blood count, peripheral smear and direct coomb's test. The second tube is a heparinised one which is used solely for osmotic fragility test and the last blood sample of 2ml is a clotted sample, collected into a plain tube which is used for indirect coomb's test and for serum bilurubin estimation**.**

**Collection of urine:**

Tem ml of fresh early morning sample of mid stream urine is collected in a sterile plastic container. The urine sample is centrifuged and the sediment is made in to smears and it is tested for hemosiderin. From that sample, 1ml sample is taken to test hemoglobinuria. After collection of samples the following panel of tests are performed to type the haemolytic anemia.

1. Complete blood count

2. Peripheral blood smear

3. Bilurubin estimation (direct and indirect)

4. Reticulocyte count.

5. Osmotic fragility test

6. Hemoglobinuria

7. Hemosiderinuria

8. Coomb's test (direct &indirect)

**CBC**

Complete blood counts for all the samples was done using SYSMEX 3 part differential analyzer and the RBC indices was noted along with total WBC count, differential count, platelet count and hematocrit.

**Peripheral blood smear**

A clean oil free glass slide and a spreader with smooth edge were taken to perform the peripheral blood smear preparation. A drop of fresh blood or EDTA blood is placed at one end of glass slide one cm from the edge and gently it was spread by the spreader at $45^0$ to the surface of the slide, and it is moved back and to make contact with the blood. Blood drop were allowed to spread along the line of contact. Smear is made with a swift movement. The film should end at least one cm away from the edge. The slide were undisturbed and allowed to dry at room temperature.

The smear is placed on two parallel glass rods which are fixed across a sink. The Leishman's stain (1.5 grams of leishman's powder is dissolved in 100 ml of acetone free methyl alcohol and allowed to ripe for 4-6 weeks) is poured onto the slide until the film is fully covered, and allowed to sit for 1-2 minutes. More stain was added to counteract evaporation. It should never be allowed to dry. Later it is diluted

with equal volume of buffered water. The diluted stain is allowed to act for 3-5 minutes, and then it is flooded with excess buffer water or with tap water. Then the under surface of the slide is wiped with a piece of filter paper. The slide is allowed to air dry.

**Bilurubin estimation**

Bilurubin is estimated by diazo method. the reagent used is Liquixx bilirubin reagent. The test was run using a blank for every sample.500 micro mi of reagent is mixed with 25 micro ml of test serum and incubated at room temperature for 5 minutes.it is compared with a standard .read the absorbance at 546/630 nm against reagent blank. Total bilurubin and conjugated bilurubin is calculated by the following formulas respectively.

Bilurubin (mg/dl) = absorbance of test x concentration of standard divided by Absorbance of standard

Direct bilurubin (mg/dl)=absorbance of the test xconcentratin of standard (mg/dl) divided by absorbance of the standard

**Reticulocyte count**

Reticulocytes are immature RBCs. They contain large amount of RNA, mitochondria and ribosome which react with some basic dyes like new methylene blue or brilliant cresyl blue to form purple precipitate of granules or filaments(fig 8). They are stained while they are alive. Therefore it is called supravital staining.

Nearly 2-3 drops of EDTA anticoagulated blood is taken in a small test tube and 2-3 drops brilliant cresyl bue stain were added to it. These are thoroughly mixed and incubate at $37^0$ for 15-20 minutes. A drop of incubated blood- stain mixture is placed on a slide and spread as a blood film and allowed for air drying. It is examined under a microscope and one thousand RBCs are counted, and the number of reticulocytes seen during the count is reported as a percentage of total number of RBC.

**Osmotic fragility test**

The red blood cell has a semi permeable membrane, through which many small substances, including water and electrolytes, can pass relatively freely back and forth. The cell has to work at keeping the normal internal electrolytic concentration (the level of potassium is much higher inside the cell than outside, while the intracellular sodium is lower than in the plasma) and to maintain its normal shape. If the cell is placed in saline solutions which are hypotonic, water from the solution will cross the semi permeable membrane into the cell where the concentration of salts are more, which will lead to the swelling of the cell (it will become more spherocytic) and ultimately it will lead to cell rupture. On the other hand, if the cells are placed in hypertonic saline, water will be drawn out of the cell and the cell will shrink or crenate. When RBCs placed in isotonic saline (.85%) there is no appreciable change in the water content of the cell occurs.

**Quantitative test**

**a) Principle**: Tubes containing solutions of varying concentrations of saline buffered to 7.4 Ph were used. Heparinised blood is added to each tube at proportion of 1:100. After the tubes have been centrifuged to settle all the RBC, the degree of haemolysis was noted after 24 hours of incubation at room temperature.

**b) Stock solution**

A stock solution of buffered sodium chloride osmotically equivalent to 10% NaCl is made up as follows:

NaCl 90 g

$Na_2HPO_4$ 13.65 g

$NaH_2PO_4 2\ H_2O$ 2.43g

Distilled water to 1000ml

The solution was stored in a stoppered bottle for further analysis and can be maintained for months without deterioration.

**Working solution**

Working solutions is prepared as 1% solution from 10% of the stock solution, by diluting in a ratio of 1:10 with distilled water. Then from this 1% solutions equivalent to 85,0.75,0.65,0.60,0.55,0.50,0.45,0.40,0.35,0.30,0.20 and 0.10 percent NaCl can be prepared. The serial concentration of saline are prepared by adding saline

and distilled water to twelve test tubes as indicated in the following table

| Drops in Distil water | 18 | 17 | 16 | 15 | 14 | 13 | 12 | 11 | 10 | 9 | 8 | 7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Drops in NACL (1%) | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
| NACL concentration | 0.28 | 0.32 | 0.36 | 0.4 | 0.44 | 0.48 | 0.52 | 0.56 | 0.6 | 0.64 | 0.68 | 0.72 |

Table – 4. Serial concentration of NaCl with distilled water.

**Method**

About 0.05 ml of heparinised blood is added to the test tubes with serial concentration of saline and incubated at room temperature. 0.05 ml control blood were added to similar set of test tubes. The test tubes were incubated overnight and then the results were read in the next morning. Normally haemolysis starts at 0.45-0.50 per cent and complete haemolysis is seen at 0.20 per cent.

**Hemosiderinuria**

Hemosiderin is an iron containing compound and it is a form in which the body stores iron. The principle of staining is the so called ‘Prussian blue reaction, in which ionic iron reacts with acid ferrocyanide to give a blue colour.

About ten ml of urine sample were taken and centrifuged at 1500 rpm for ten minutes. The supernatant is poured off. Nearly 1-2 drops of the sediment is placed on glass slide and a smear is made as per the procedure followed earlier. It was allowed to air dry. Then it was fixed with methyl alcohol. The slide was placed on two parallel glass rods which are placed above a plastic tray. Then 5 ml of a staining mixture

obtained by mixing equal volumes of 2% potassium ferrocyanide and 2% HCl was poured over the slide and allowed to stand for 10-15 minutes. Then the slide was air dried and mounted with DPX. Typically deeply blue stained granules( fig.5&6) indicate a positive test result.

**Hemoglobinuria**

Blood or haemoglobin excreted in urine is detected by benzidine test. The principle is an enzymatic reaction where the haemoglobin peroxidase act on $H_2O_2$ to liberate nascent oxygen. Oxygen oxidises benzidine to a blue coloured compound.

About 2ml of glacial acetic acid was taken , a pinch of Benzedrine powder was added in a test tube. Five ml of urine and 1ml of hydrogen peroxide was added to this test tube. A colour change to blue marks the test as positive.

**Coomb'test**

**DIRECT ANTIGLOBULIN TEST (DAT)**

**Method**

The test tubes were labelled with the respective patients name. About 2-3 drops of patent's red cells were placed in the tube and then washed 3 times with saline to remove all the traces of serum. A drop of coombs serum was added to the tube, and kept at room temperature for 5 minutes. Then the contents were centrifuged slowly for 1 minute. A drop from the test tube were taken and placed it a glass slide and covered with the cover slip and the slides were subjected for macroscopic examination. Agglutination indicates positive result.

## Indirect antiglobulin test (IAT)

### Method

The pooled ‘o’ cells are obtained from the blood bank and were washed three times in normal saline. About 5 per cent saline suspension of pooled ‘o’ cells were prepared. Three test tubes were taken and labelled as T which represents the test serum; PC for Positive control and NC for negative control. In the tube labelled as ‘T ’ 2 drops of test serum was added , in test tube labelled as PC two drops of Anti-D serum was added. One drop of saline is added in the tube labelled as ‘NC’. In each tube one drop of 5 % saline suspension of the pooled ‘O’ cells was added. The tubes were incubated for one hour at 37°C. Then 2 drops of Coombs serum was added to each tube and kept for 5 minutes and then centrifugde at 1,500 RPM for one minute. The cells are resuspended and examined for agglutination under microscope. Agglutination in both test and control and no agglutination in negative control indicate a positive result.

# RESULTS AND OBSERVATION

DIAGNOSIS OF HAEMOLYTIC PROCESS USING PERIPHERAL SMEAR

In our study group out of 14 cases 7 were diagnosed as thalassaemia which forms 50% of our study group. Among the infants one was diagnosed as haemolytic disease of newborn which constitutes about 7.14% of our study population, 3 were assigned as post haemolytic and 3 were categorized as others which constitutes about 21.42% each of the study population.

TABLE . 1 MORPHOLOGICAL ANALAYSIS OF HAEMOLYTIC ANEMIAS

| Diagnosis | No. of cases |
|---|---|
| Thalassaemia | 7 (50%) |
| HDN | 1 (7.14%) |
| Post hemolytic anemia | 3 (21.42%) |
| Others | 3 (21.42%) |
| Total | 14 |

ACUTE LYSIS

In the study group out of 14 cases 1 patient had hemoglobinuria. It constituted about 7.14%.It was a new born female baby which had jaundice inappropriate to the haemolytic process.

TABLE .2: HAEMOGLOBINURIA POSITIVITY (ACUTE LYSIS)

| Total No. of cases | HBN Positive | HBN Negative |
|---|---|---|
| 14 | 1(7.14%) | 13(92.86%) |

CHRONIC HEMOLYSIS (HEMOSIERINURIA AMONG STUDY GROUP)

HEMOSIERINURIA AMONG STUDY GROUP

Among the group studied out of 14 patients 7 were found hemosiderinuria positive and the remaining 7 were hemosiderinuria negative which constitutes about 50% each of the studied population. Of the 7 positive cases 6 were thalassaemics and one is assigned as post haemolytic.

TABLE 3 CHRONIC HAEMOLYSIS

| Hemosiderinuria | Positive | Negative | Total |
|---|---|---|---|
| No. of Cases | 7(50%) | 7(50%) | 14 |

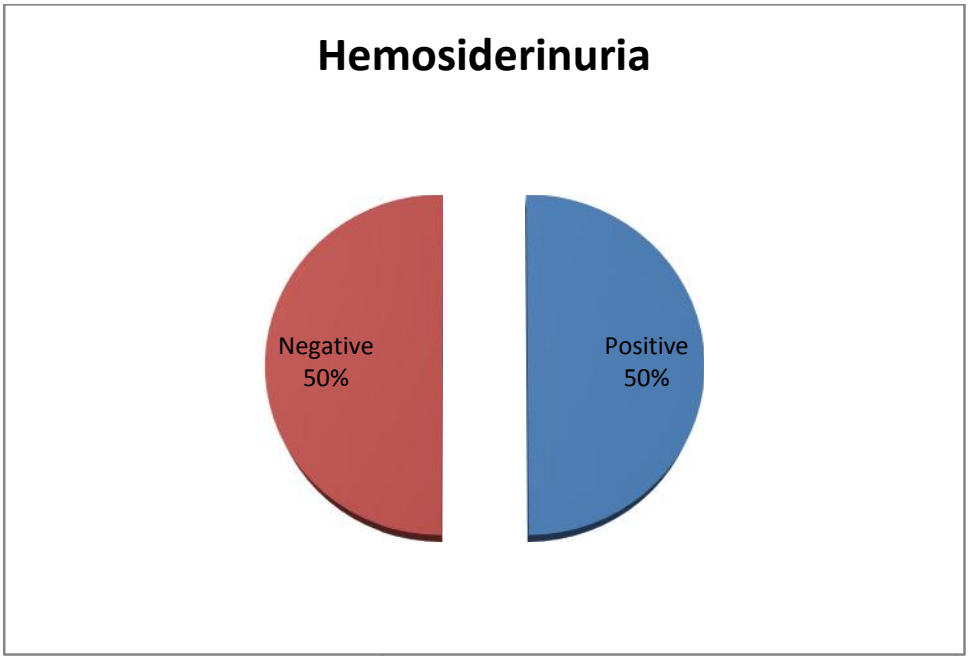
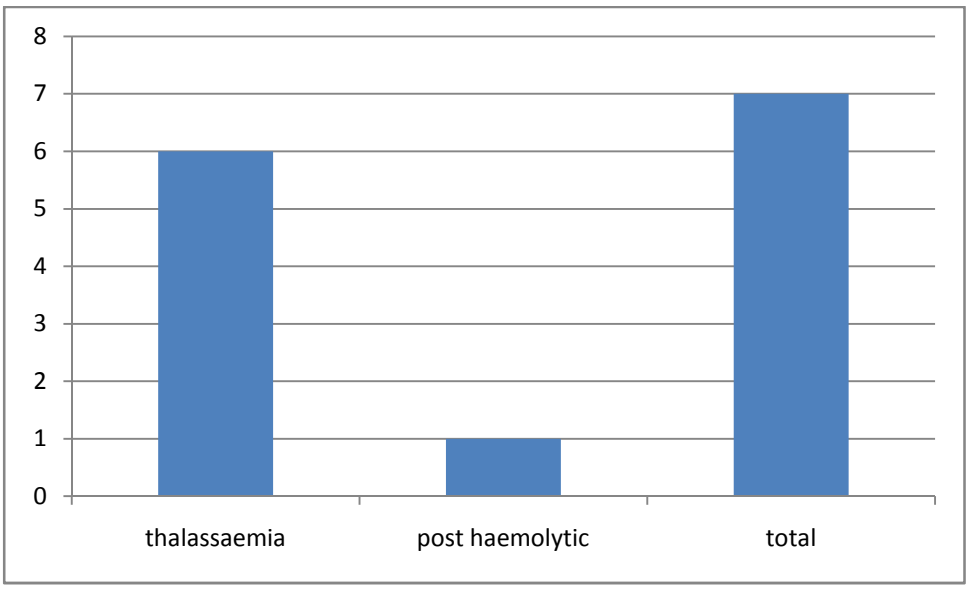



CHART NO.1: HEMOSIERINURIA AMONG STUDY GROUP

TABLE 4: HAEMOSIDERINURIA POSITIVITY AMONG VARIOUS CASES

| | Thalassaemia | Post haemolytic | total |
|---|---|---|---|
| HSDN positive | 6 (42.85) | 1(7.14%) | 7 |

CHART NO 4 : HAEMOSIDERINURIA POSITIVITY AMONG VARIOUS CASES




IMMUNE HAEMOLYSIS

Among the study group no case was found to be DCT or ICT positive. So the percentage of immune hemolytic anemia among study group is zero percentage.

TABLE.5

| Total No. of cases | DCT Positive | ICT Positive |
|---|---|---|
| 14 | 0 | 0 |

TABLE.6 RED CELL LYSIS (OSMOTIC FRAGILITY TES

| Total No. of cases | Normal | increased | Decreased |
|---|---|---|---|
| 14 | 8(57.14%) | 1(7.14%) | 5(35.71%) |

In the study group out of 14 cases 8 had a normal osmotic fragility test. One patient had increased osmotic fragility and 5 patients had decreased osmotic fragility which constitutes about 7.14% and 35.71% respectively.

INDEX OF ACUTE HEMOLYSIS
(RETICULOCYTE COUNT)

Among the group studied the reticulocyte count was found increased in 11 patients i.e. 78.57 % of patients had increased reticulocyte count. One patient had normal and another two patients had a decreased reticulocyte count which constituted about 7.14% and 14.28% respectively.

TABLE.7: RED CELL LYSIS

| Total No. of cases | normal | increased | decreased |
|---|---|---|---|
| 14 | 1(7.14%) | 11 (78.57) | 2 (14.28%) |

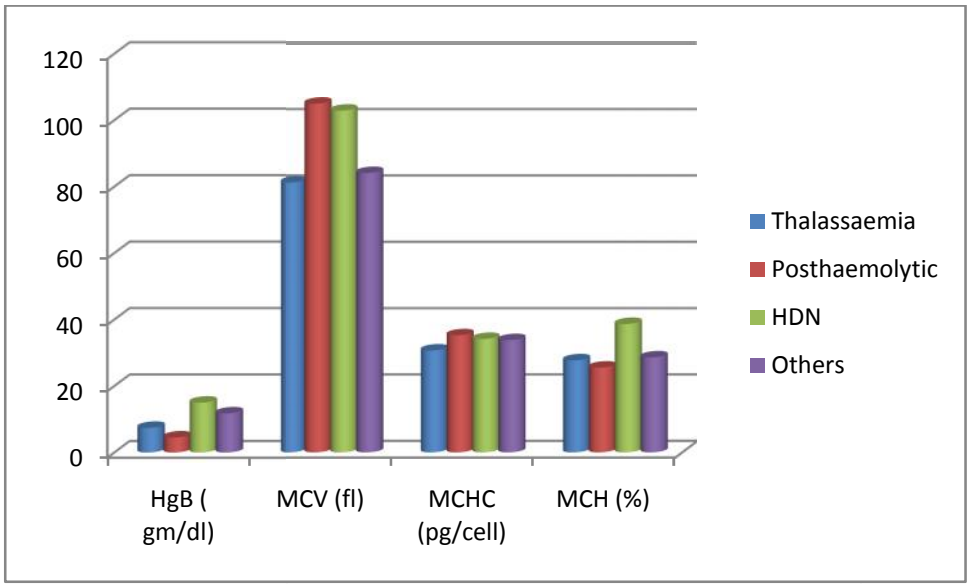
RED CELL INDICES IN VARIOUS HAEMOLYTIC ANEMIAS

The mean values of RBC indices in thalassaemia is as follows: Hb 7.42, MCV 81.3, MCH 27.74, MCHC 30.7 ,in post hemolytic state is as follows : Hb 4.53,MCV 105.1, MCH 25.56, MCHC 35.3 and in the group assigned as others is Hb 11.76 MCV 84.2, MCH 28.63, MCHC 33.83. In a case of HDN the red cell indices are Hb 15,MCV 103,MCHC34.2 andMCH38.7.

TABLE 8: RED CELL INDICES IN VARIOUS HAEMOLYTIC ANEMIAS

| **RED CELL INDICES IN HAEMOLYTIC ANEMIA** | | | | |
|---|---|---|---|---|
| | HgB ( gm/dl) | MCV (fl) | MCHC (pg/cell) | MCH (%) |
| Thalassaemia | 7.42 | 81.3 | 30.7 | 27.74 |
| Posthaemolytic | 4.53 | 105.1 | 35.3 | 25.56 |
| HDN | 15 | 103 | 34.2 | 38.7 |
| Others | 11.76 | 84.2 | 33.83 | 28.63 |

CHART NO 5: RED CELL INDICES IN VARIOUS HAEMOLYTIC ANEMIAS



RESULTS OF BIOCHEMICAL PARAMETER OF LYSIS

In our study group among 14 cases bilirubin found elevated in 13 patients and it was found normal in one patient.

TABLE 9: RESULTS OF BILIRUBIN

| Total No. of cases | Decreased | increased | Normal |
|---|---|---|---|
| 14 | 0 | 13 (92.85%) | 1 (7.14%) |

TABLE 10:

| Intravascular haemolytic anaemia | Extravascular haemolytic anaemia |
|---|---|
| 8 (57.14%) | 6 (42.85%) |

DIAGNOSIS OF HAEMOLYTIC PROCESS USING THE SCREENING PANEL

In our study group out of 14 cases 7 were diagnosed as thalassaemia which forms 50% of our study group.among the infants one was diagnosed as haemolytic disease of newborn which constitutes about 7.14% of our study population,3 were assigned as post haemolytic and 3 were categorized as others which constitutes about 21.42% each of the study population.

TABLE 11. DISTRIBUTION OF VARIOUS HAEMOLYTIC ANEMIAS

| Diagnosis | No. of cases |
|---|---|
| Thalassaemia | 7(50%) |
| HDN | 1(7.14%) |
| Post hemolytic anemia | 3(21.42%) |
| Others | 3(21.42%) |
| Total | 14 |

CHART NO 10: DISTRIBUTION OF VARIOUS HAEMOLYTIC ANEMIAS

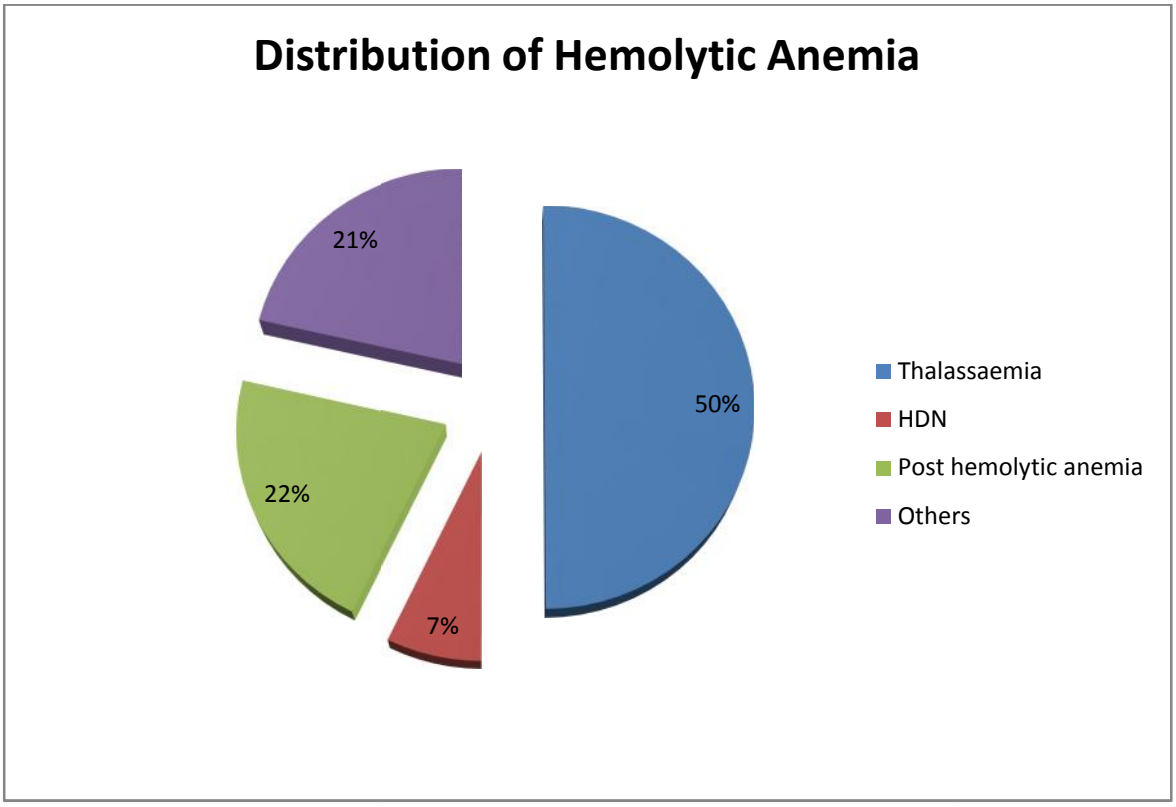



TABLE.12 Efficacy of screening panel

| Diagnosis | Disease | Others | statistics |
|---|---|---|---|
| Yes | 6 | 0 | P =0.01 |
| No | 2 | 6 | |

Table 11 shows the efficacy of screening panel in diagnosing haemolytic anemias. Of the 14 cases 6 were already diagnosed,2 were newly diagnosed and 6 were could not be categorized. Fisher's exact test reveals that the test is statistically significant with a p value of 0.01.

TABLE.13 Acute Hemolysis

| | Diagnosis | |
|---|---|---|
| Results | Disease | others |
| Positive | 0 | 1 |
| Negative | 11 | 2 |

Table 17 shows efficacy of haemoglobinuria in acute haemolysis. Of which 1 came out to be positive in the others group, rest of them were negative. Fisher's exact test reveals that the test was statistically insignificant with a p value of 0.214.

TABLE.14: Chronic Hemolysis

| | Diagnosis | |
|---|---|---|
| Results | Disease | others |
| Positive | 7 | 0 |
| Negative | 2 | 5 |

Table 13 shows efficacy of haemosiderinuria in chronic haemolysis. Of the 14 variables 7 denotes positive in categorized group,2 denotes negative in categorized group and 5 denotes negative in others group. Fisher's exact test reveals that the test was statistically significant with a p value of 0.021.

TABLE 15. Red Cell Lysis

| Diagnosis | | |
|---|---|---|
| | Disease | others |
| Normal | 6 | 2 |
| Increased | 0 | 1 |
| Decreased | 5 | 0 |

Table no 14 shows the efficacy of osmotic fragility test. Of the 14 variables 6 denotes categorized cases with normal OFT,5 denotes categorized cases with decreased OFT,2

denotes others group with normal OFT and 1 denotes others group with increased OFT. Pearson chi square test revealed a p value of 0.078. With more number of samples the efficacy can be better.

TABLE 16. Reticulocyte index

| | Diagnosis | |
|---|---|---|
| | Disease | others |
| <0.5 | 1 | 1 |
| 0.5-2.5 | 1 | 0 |
| >2.5 | 9 | 2 |

Table no 15 shows the efficacy of reticulocyte count in red cell lysis. Of the 14 variables 1, 1, and 9 denotes decreased, normal and increased reticulocyte count in categorized group 1, 2, 0 of the others group represent decreased, increased, and normal reticulocyte count. Pearson chi square test revealed a p value of 0.519. With more number of samples the efficacy can be better.

TABLE 17 : Biochemical evidence of haemolysis

| | Diagnosis | |
|---|---|---|
| Bilirubin | Disease | others |
| <1.5 | 1 | 0 |
| 1.5-3 | 2 | 0 |
| >3 | 8 | 3 |

Table no 16 shows the efficacy of level of bilurubin in diagnosing haemolysis. Of the 14 variables 1, 2, and 8 denotes normal, moderate, and severe increase in bilirubin level in categorized group 0, 0 and 3 of the others group represent normal, moderate

and severe increase bilirubin level. Pearson chi square test revealed a p value of 0.594. With more number of samples the efficacy can be better.

**Discussion**

The study was conducted in Tirunelveli medical college hospital, tirunelveli, southern Tamilnadu, suspected cases of haemolytic anaemia who were referred to the clinical haematology lab were included in the study. A total number of 14 patients were subjected to the screening panel.

With regard to clinical features most of the patients presented with moderate to severe anaemia, icterus, and splenomegaly. Of the 14 patients two newborns presented with normal haemoglobin range but with other features of haemolysis.

In our study, majority of patients were paediatric i.e. 64% while remaining 36% constitute adult population .With regard to sex distribution it was equal. R.H.Deshpande and A.R.Joshi et .al study on haematological profile in haemolytic anemia [101] in the region of marathwada, Maharastra showed 67.1% were males and remaining 32.8% were females.

R.H.Deshpande and A.R.Joshi et .al showed similar pattern of distribution of haemolytic anemias dominated beta thalassaemia (41.17%) followed by other haemoglobinopathies. but in their study they included Haemoglobin electrophoresis which enabled to them to diagnose better of the suspected cases. In our study our vital tool for diagnosis is peripheral smear. With peripheral smear alone we can be able to categorise cases as thalassaemia (50%),haemolytic disease of newborn(7.14%) ,post haemolytic state (21.42%) and the remaining were assigned as others for which no

conclusive clue derived from peripheral smear (table no.1). In the others group one case was suspected to be of enzyme defect.

Out of 14 patients one sample was positive for haemoglobinuria and others were found to be negative (table .2). haemoglobinuria was positive in a new born. The baby had severe jaundice with elevated WBC count of 1, 20,800 with a clinical suspicion of a haematological malignancy. But peripheral smear examination showed elevated nRBCS revealing an underlying haemolytic process. So the haematological malignancy was ruled out but it could not be categorised on the basis of screeninig panel.

Haemosiderinuria was found positive in 50% of cases the rest were found negative (tab.no.3). Of the positive cases 6 were thalassaemics (42.85%) and one case was categorised as post haemolytic state. haemosiderinuria was taken as an index of chronic haemolysis in the present study. Fisher's exact test was done (table 14).

In our study all our patients were subjected to osmotic fragility test. It was found normal in 57.14% of cases, increased in 7.14% of cases and decreased in 35.71 % of cases (table no .6). The case in which it was found increased was categorized on others because the peripheral smear showed microspherocytes of variable size. With increased reticulocyte count and elevated bilurubin, decreased MCV, decreased MCH and a normal MCHC. Coomb's test was negative. So the case may be an acquired spherocytosis but could not conclude based on the screening panel. In 6 cases of thalassaemia the OFT was found decreaed i.e resistant osmotic lysis. In rest of the cases the haemolysis started between 0.4 to 0.5 % concentrations of NaCl. The group

which showed normal include 4 caes of thalassaemia, one categorized as post haemolytic state and a case of enzyme defect.

All the samples were subjected to reticulocyte count as a part of our screening panel which showed an increased count in 78.57% of cases (table no.7).the normal and decreased count category had moderate anemia,with mild elevation of bilurubin . We were able to diagnose a case of α-thalassaemia based on studying the smear and the typical 'golf ball' appearing reticulocytes( fig.4) were made out.

in our study by analysising our panel of investigations we were able to assign 57.14% of patients are having intravascular haemolysis and 42.85% having extravascular haemolysis.

Gurpreet, Dhaliwal, et al [102]and R. J. Abbott et al.[103] showed hyperbilurubinemia is associated with haemolytic disorders. In our study group 92.85% of population had an increased level and 7.14% had a normal level (table no.9). This correlate with other studies of hyperbilurubinemia and an underlying haemolytic pathology. In a study on thalassaemias Amirta panja et al showed MCV of 50-69fl,MCH of 12-30 pg and Hb of 7-11 gm .Comparing with the above study our study showed a MCV of 81.3 which is slightly higher ,Hb of 7.42 which is similar to the above study. MCH is 27.74 which is also similar to the above study. The post haemolytic group showed an average Hb of 4.53gm/dl,mcv of 105.1fland mch of 25.56 pg. the group asssingned as others showed an average Hb of 11.76,mcv of 84.2 and MCHC of 33.83pg/cell.

Giri et al at boroda (Gujrat) in 1984 and Joshi Anil et al in (1986), R.H.Deshpande and A.R.Joshi et .al . showed thalassaemias were the most common

haemoglobinopathy followed by sickle cell anemias. But our study group out of 14 cases 43 % were already diagnosed cases of thalassaemia on follow up we applied our panel of tests to test its efficacy. We were able to diagnose a case of alpha thalassaemia based on our screening panel. This is lesser than earlier studies like R.H.Deshpande and A.R.Joshi et .al . But we have not applied any diagnostic tests like haemoglobin electrophoresis or gel electrophoresis which would have made it possible for categorising more cases. We were able diagnose a case of HDN using this screening panel.in 21.42% of cases the predominant feature was microspherocytes with other parameters like elevated bilirubin and reticulocytes which favoured a post haemolytic state and further investigations are warranted for accurate diagnosis. The remaining 21.42% we were able to establish an underlying haemolytic process.

Rao K Uma Maheswara, Manoj Y et al [104] study of Clinico-haematological Profile of Haemolytic Anaemias in 36 cases ,Andhra Pradesh, India showed Sickle cell anaemia-7 cases (19.44%), Sickle cell trait- 1 case (2.77%), Sickle cell/beta thalassemia-12 cases (33.33%), betathalassemia major- 13 cases (36.11%) and beta thalassemia intermedia 1 case (2.77%). Sickle beta thalassemia and sickle cell anaemia combinely constitutes 53% of cases followed by beta thalassemia major. Haemolytic anaemias with enzyme defects and membrane defects were not observed in this study. Majority of these cases presented with progressive pallor and hepato splenomegaly. Peripheral blood smear examination showed microcytic hypochromic anaemia in majority of the cases. All cases were associated with reticulocytosis. Haemoglobin electrophoresis confirmed the diagnosis. The clinical presentation, reticulocyte count and PBF findings are similar to our results. In our study we had

similar distribution dominated by haemoglobinopathies. We were not able to diagnose membrane defects or enzyme defects with our panel of investigations as like the bove mentioned study.. In contrast to the above study our work up does not include haemoglobin electrophoresis.

Kaustubh Chattopadhyay , Romy Biswas et al [105]an epidemiological study on the clinico – hematological profile of patients with congenital hemolytic anemia in a tertiary care hospital of Kolkata showed the commonest congenital hemolytic anemia in the present study is HbE Beta Thalassemia (41.1%) followed by Beta Thalassemia (23.6%) total WBC count, RBC count ,differential WBC count, evaluation of platelets, Serum total bilirubin, Serum Ferritin and Hb Electrophoresis were done in this study. The results were similar to our study except Hb Electrophoresis and Serum Ferritin which will be useful to make a better diagnosis.

This basic screening panel includes simple tests which are time saving and cost effective. With this panel of investigations we can be able make out whether the etiology of haemolysis whether acute or chronic or immune mediated. By combining red cell indices, peripheral amear and reiculocyte count most of the etiology of haemolytic process can be predicted. Other tests like osmotic fragility test will give direction to further work up. Ayalew Tefferi et al [98]in their study of A Contemporary Approach to Diagnosis in Anemia in Adults showed the significance of haemosiderinuria with regards to exravascular and intravascular causes of haemolysis. In our study 50 % of study group showed hemosiderinuria. The efficacy of haemosiderinuria in chronic haemolysis as per fisher exact test was 0.021 which was proved to be a significant index in our panel.

The efficacy of the panel will be better if the samples were received before the commencement of therapy. The efficacy of the screening panel will be better if the sample size is large.

## SUMMARY

In our study we subjected a group of patients with suspected clinical diagnosis of haemolytic anemia to a basic panel of investigations. With peripheral smear analysis we assigned 50% of cases as Thalassaemias, 21.42% as post haemolysis, 7.14% as haemolytic disease of new born 21.42% as others.

Haemoglobinuria testing in our study group we had found 7.14% were positive and 92.86% were found negative. Haemosiderinura was found to be positive in 50% of cases and negative in 50% of cases. The efficacy of haemosiderinuria testing was found to more significant. In our study group DCT & ICT were found to negative in all patients. The osmotic fragility test was found to be increased in 7.14% of cases, decreased in 35.71% of cases and it was found normal in 57.14% of cases. The reticulocyte index which is an index of acute haemolysis was found to be increased in 78.57% of cases, decreased in 14.28% of cases and it was normal in 7.14% of cases.

Bilirubin, a biochemical parameter of haemolysis was found elevated in 92.85% of cases and was found normal in 7.14% of cases which proves to be a significant biochemical index of haemolysis.

We were able to assign 57.14% as intravascular haemolytic anaemia and 42.85% as extravascular haemolytic anaemia.

Over all as a basic screening panel it is efficient in making a diagnosis 50% of patients were having thalassaemia, 7.14 % of patients were having haemolytic disease of newborn, 21.42% were diagnosed as post haemolysis and the remaining 21.41% are assigned as others in whom an underlying haemolytic process was established by

using our panel. The efficacy of screening panel was significant with a ‘p’ value of 0.01. From this study, 79% of patients having a clinical suspicion of haemolytic anaemia were diagnosed using basic screening panel of investigations.

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## ANNEXURE I

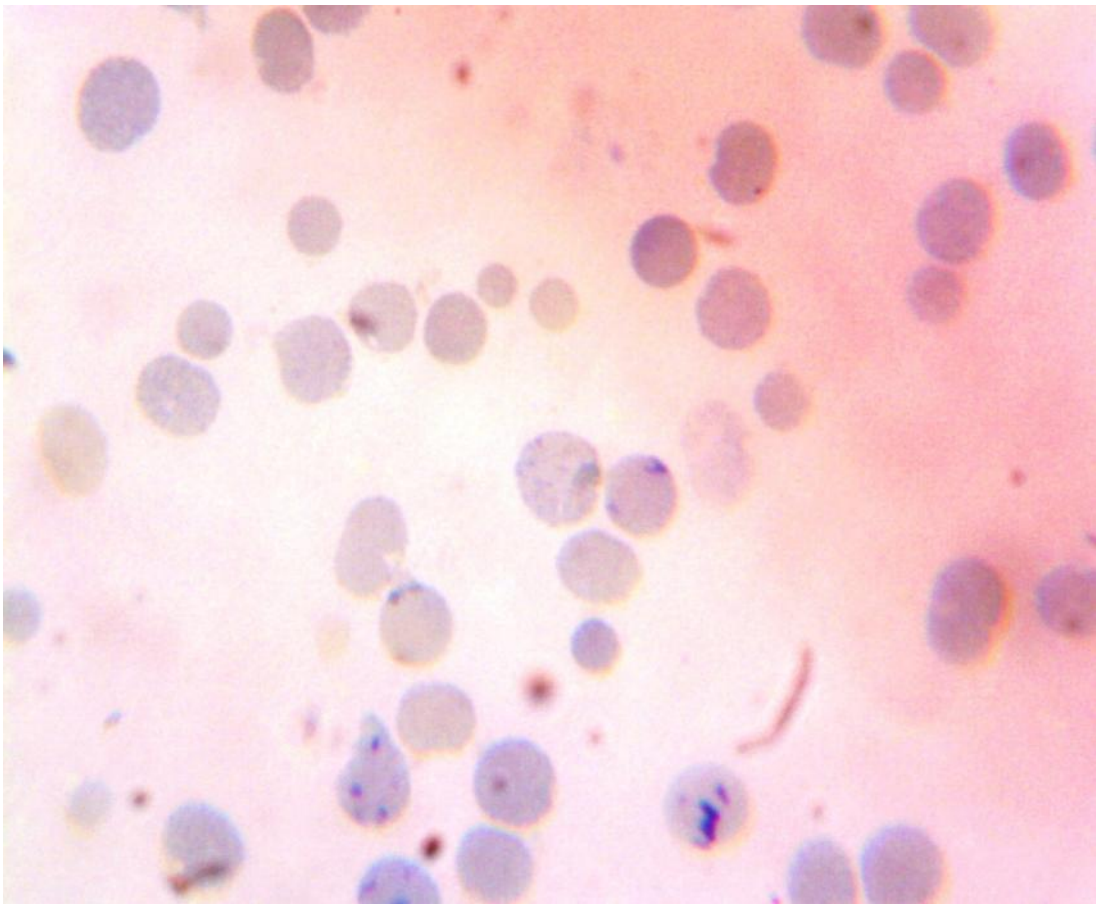

**Figure – 4**. Golf ball appearance of Reticulocytes  seen in HbH.

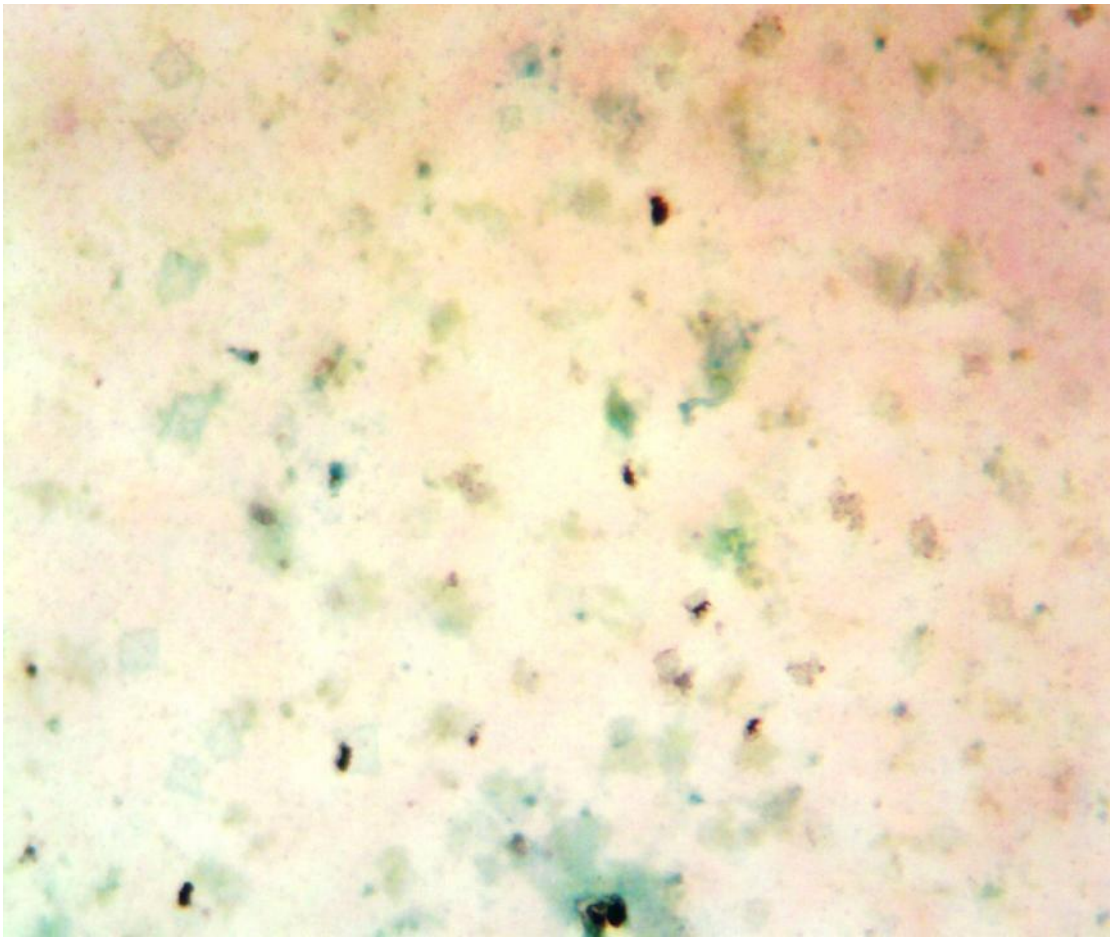

**Figure – 5**. Hemosiderin deposits in urine (Prussian Blue stain).

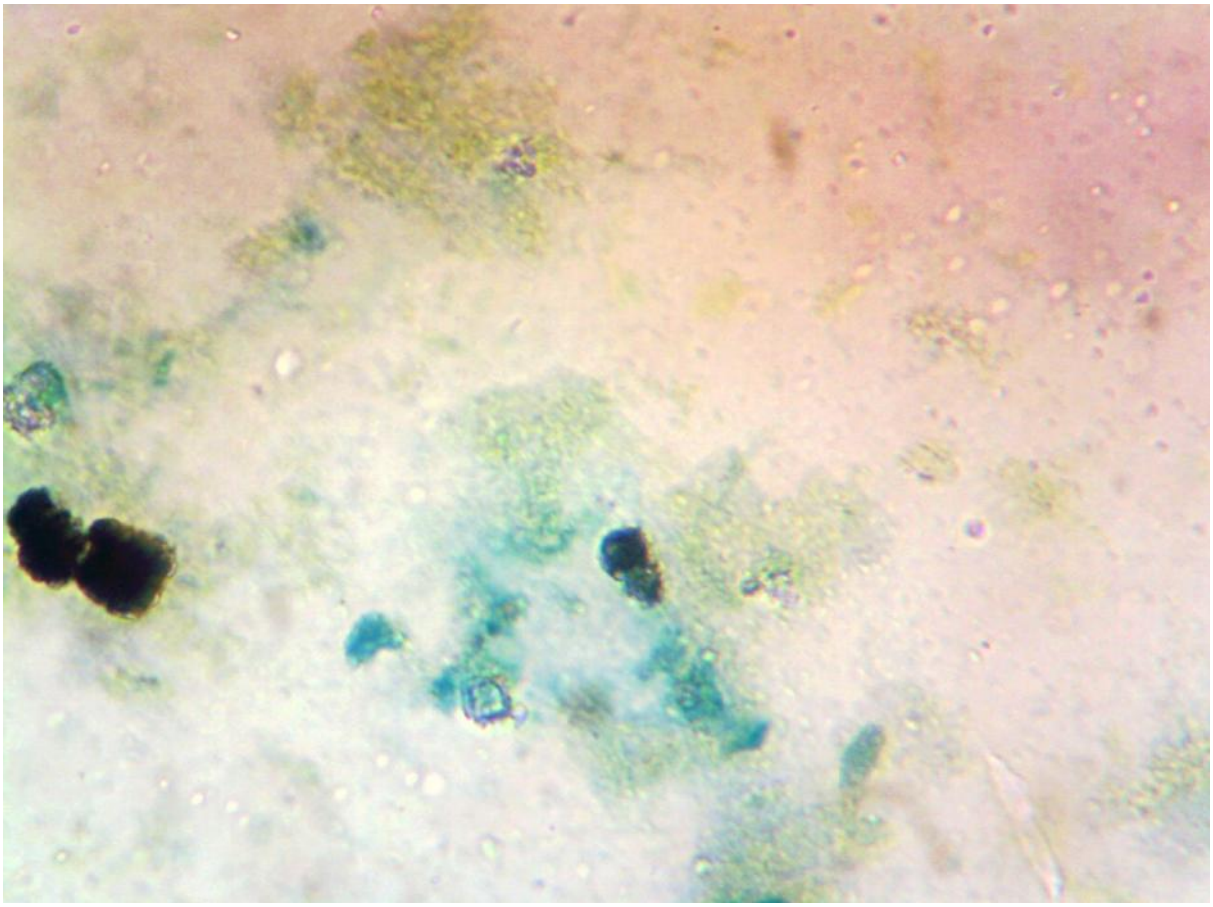

**Figure – 6.** Hemosiderin deposits in urine (Prussian blue stain).

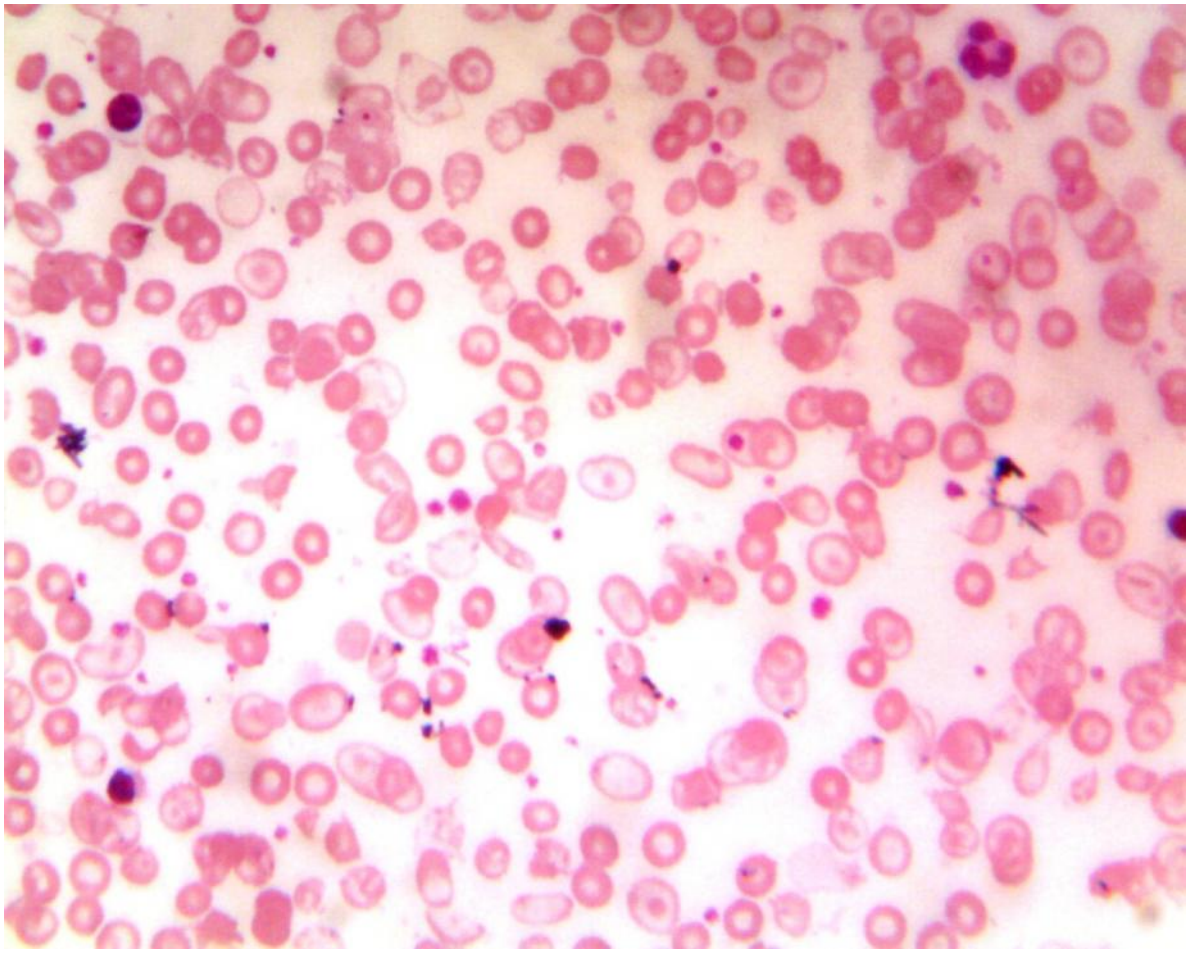

Figure – 7. Target cells, microcytes, ovalocytes, nRBCs, and elliptocytes (Leishman stain).

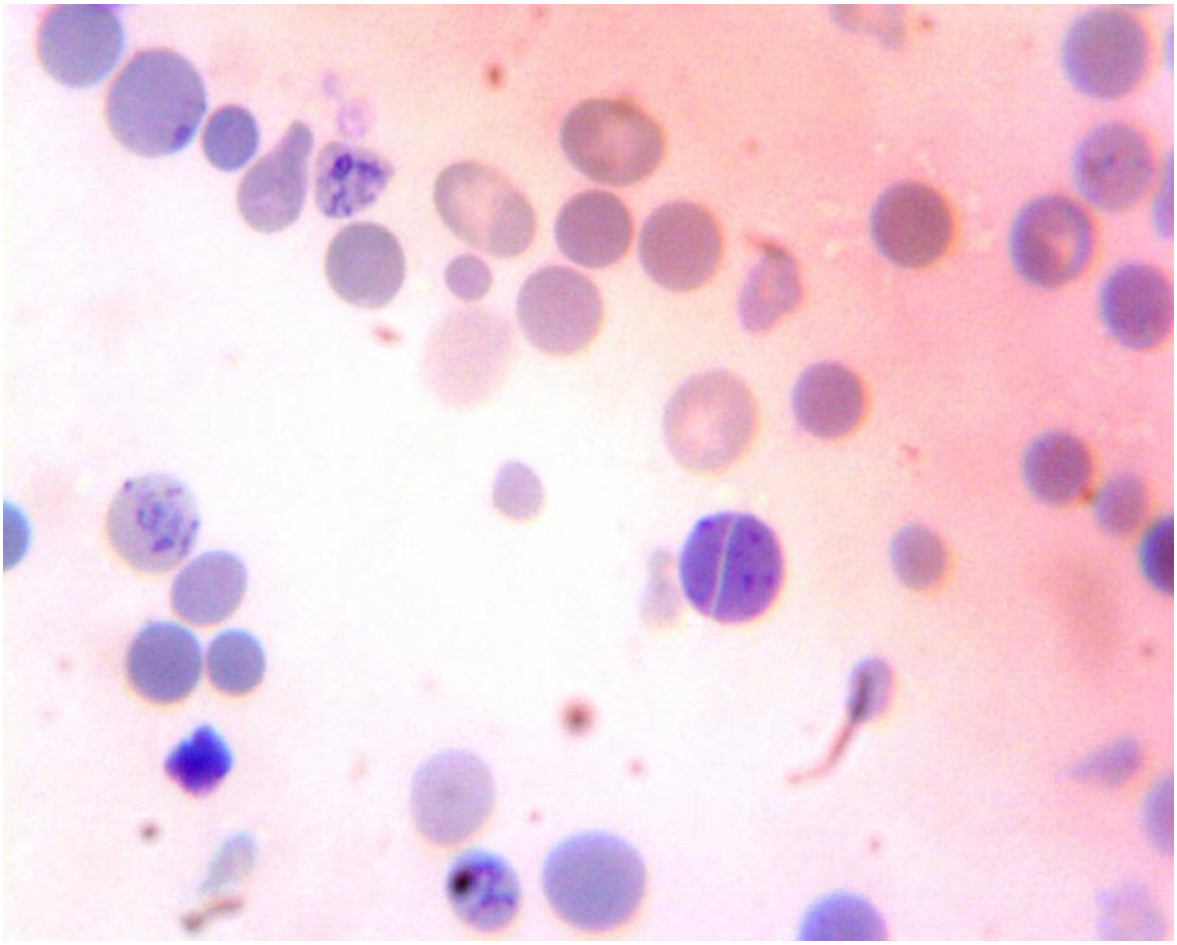

Figure – 8. Reticulocytes showing bluish thread like material (supravital stain).

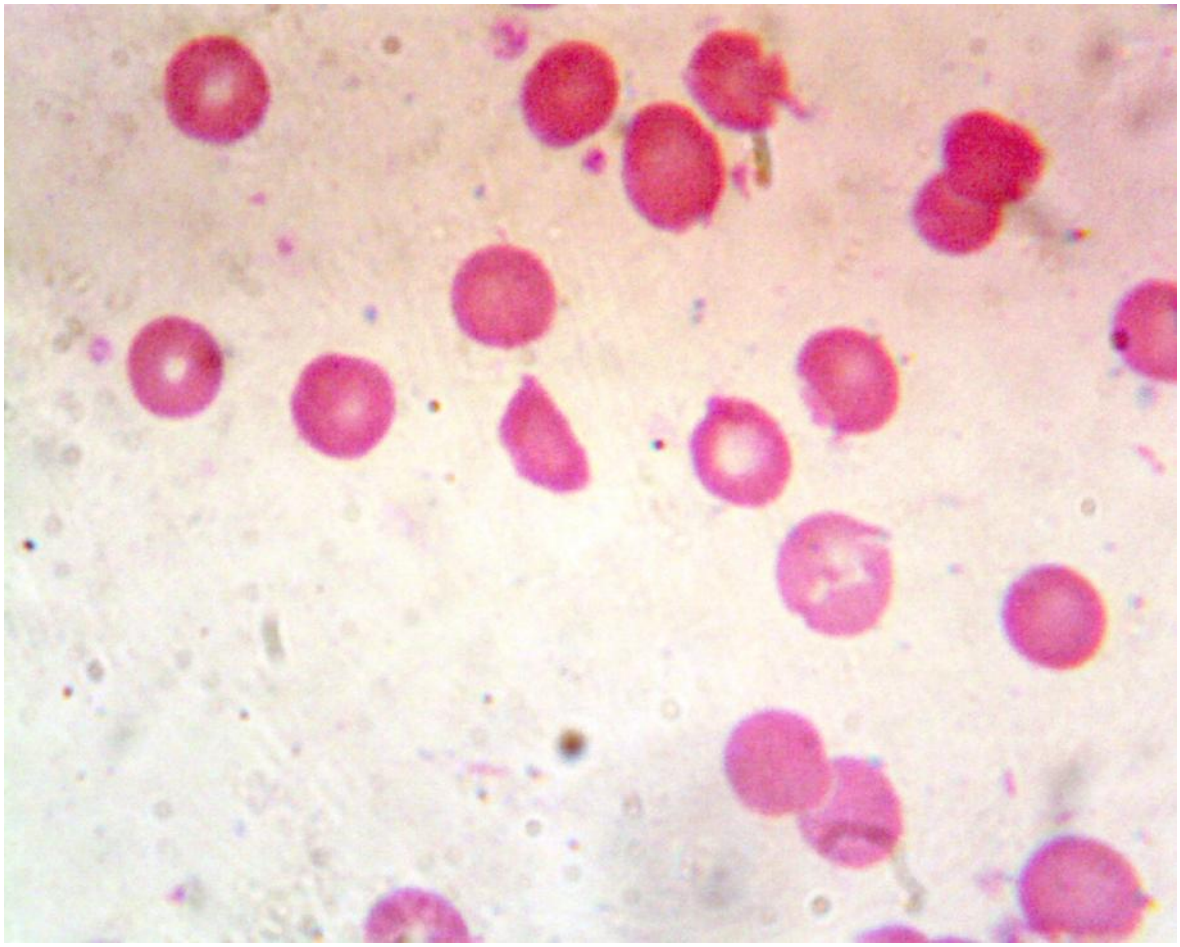

Figure – 9. Showing microspherocytes.

## ANNEXURE II

## PROFORMA

**NAME**

**AGE**

**SEX**

**ADDRESS**

**OCCUPATION**

**DURATION AND DETAILS OF ILLNESS**

**SYMPTOMS**

- Easy fatiguability
- Frequent falls
- Dizziness
- Tinnitus
- Palpitations
- Abdominal distention
- Symptoms of thyroid disturbances
- Recurrent infections

**PAST HISTORY**

- Diabetes
- Hypertension
- Coronary artery disease
- Tuberculosis
- Malignancies
- Chronic kidney disease

**DRUG HISTORY**

**PERSONAL HISTORY**

- Smoking
- Alcoholism

**GENERAL EXAMINATION**

- Pallor
- Icterus
- Pedal edema
- Lymphadenopathy
- Clubbing
- Koilonychia
- Glossitis
- Angular stomatitis
- Aphthous ulcers

**SYSTEMIC EXAMINATION**

**CVS**

**RS**

**ABDOMEN**

**CNS**

**INVESTIGATIONS**

- CBC
- ESR
- Stool for occult blood
- Blood urea

- Serum creatinine
- Liver function tests
- Peripheral smear
- Reticulocyte count
- Serum ferritin
- Serum iron
- Total iron binding capacity
- Imaging studies
- Endoscopic studies ( if needed)

## ஆராய்ச்சி தகவல் தாள்

திருநெல்வேலி மருத்துவ கல்லூரி அரசு பொதுமருத்துவமனைக்கு வரு நோயாளிகளுக்கு குருதிசிதைவு இரத்தசோகை நோய் பற்றிய ஒரு ஆராய்ச்சி நடை பெற்றுவருகிறது.

குருதிசிதைவு இரத்த சோகை என்பது எவ்வளவு பரவலாக இருக்கிறது என்பது பற்றியும், அதற்கான காரணங்கள் என்ன என்பது பற்றியும் அறிந்து கொள்வதே இந்த ஆராய்ச்சியின் நோக்கமாகும்.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்புகிறோம். முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடை விருப்பத்தின் பேரில்தான் இருக்கிறது . மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த சிறப்புப் பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவில் தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக் கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி:

# ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சிதலைப்ப : எளிய பரிசோதனைகள் மூல குருதிசிதைவு இரத்தசோகை நோய் வகைபடுத்துதல பற்றிய ஆராய்ச்சி .

பெயர்: தேதி:

வயது : உள்நோயாளிஎ :

பால் : ஆராய்ச்சி சேர்க்கை எ :

இந்த ஆராய்ச்சியின் விவரங்கள அதல் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது. எனக்க விளக்கப்பட்ட விஷயங்களை நால் புரிந்து கொண்டு எனது சம்மதத்தை தெரிவிக்கிறேன்.

இரத்தசோகை அறிகுறிக மற்றும் பாதிப்புக குறித்து ஆராய்ச்சியாளர் கூ முழுவதும் விளங்க பெற்றேன்.

இதற்குத் தேவையான உடற்பரிசோதனைக்கும், இரத்தம் சம்பந்தப்பட்ட பரிசோதனைகளுக்கும் மனமார சம்மதிக்கிறேன்.

கையொப்பம்

## *MASTER CHART*

| Sl. No | A | S | T C | D C | | | RBC | PCV | HB | MCV | MCHC | MCH | platelet count | R.C | Bilirubin | O.F.T | coombs test | | HSDN | HBU | P.S. Diagnosis |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | P | L | M | | | | | | | | | | | D | I.D. | | | |
| 1 | 8 | M | 9400 | 30 | 67 | 3 | 2.47 | 19.6 | 7 | 79.4 | 35.7 | 13 | 45000 | 3% | 3.1 | 0.4 | Neg | Neg | Pos | Neg | thal major |
| 2 | 7 | M | 7400 | 61 | 34 | 5 | 2.26 | 18.8 | 6.6 | 83.2 | 35.1 | 34.1 | 212000 | 4% | 1.6 | 0.4 | Neg | Neg | Pos | Neg | thal major |
| 3 | 11 | F | 5900 | 48 | 48 | 4 | 2.56 | 11.2 | 3.3 | 55.9 | 23.1 | 32.9 | 112000 | 3% | 3.2 | 0.5 | Neg | Neg | Neg | Neg | thal major |
| 4 | 6/356 | FCH | 37200 | 18 | 80 | 2 | 4.33 | 43 | 15 | 101 | 33 | 34.1 | 58000 | 24% | 12.6 | Nor | Neg | Neg | Neg | Pos | others |
| 5 | 15 | m | 7200 | 50 | 32 | 18 | 4.6 | 34.2 | 12 | 73.2 | 35.4 | 25.9 | 206000 | 0.40% | 3.3 | n | Neg | Neg | Neg | Neg | others (enzyme defect |
| 6 | 2 | m | 15000 | 61 | 23 | 17 | 3.2 | 21.1 | 8.3 | 78.4 | 33.1 | 25.9 | 77000 | 3% | 5.2 | ↑0.8 | Neg | Neg | Neg | Neg | A.Sperocytosis |
| 7 | 20 | f | 10300 | 45 | 46 | 9 | 0.84 | 8.6 | 3 | 102 | 34.8 | 35.7 | 220000 | 4% | 8.7 | n | Neg | Neg | Neg | Neg | post hemolytic |
| 8 | 25 | f | 3100 | 44 | 51 | 5 | 2.2 | 9.1 | 2.8 | 126 | 38.9 | 12.7 | 41000 | <0.5% | 3.2 | 0.5 | Neg | Neg | Neg | Neg | post hemolytic |
| 9 | 2/365 | f | 46312 | 22 | 72 | 6 | 3.92 | 45 | 15 | 103 | 34.2 | 38.7 | 44000 | 17.50% | 5.2 | 0.5 | Neg | Neg | Neg | Neg | HDN |
| 10 | 4 | m | 8100 | 37 | 59 | 3 | 2.5 | 22 | 9.5 | 78.4 | 31 | 38 | 220000 | 5% | 1.2 | n | Neg | Neg | Pos | Neg | thal inter |
| 11 | 36 | m | 6400 | 62 | 33 | 5 | 2.7 | 32.2 | 8.5 | 119 | 26 | 31.4 | 750000 | 11% | 3 | n | Neg | Neg | Pos | Neg | HbH |
| 12 | 10 M | F | 83000 | 37 | 63 | 0 | 2.8 | 20.9 | 6.1 | 74 | 29.1 | 21.7 | 152000 | 7.50% | 4.6 | n | Neg | Neg | Pos | Neg | thal major |
| 13 | 4 | f | 15600 | 38 | 53 | 8.2 | 3.83 | 30.4 | 11 | 79.4 | 35.5 | 23.1 | 92000 | 2% | 4.1 | n | Neg | Neg | Pos | Neg | thal inter |
| 14 | 52 | m | 27000 | 57 | 38 | 4.5 | 2.76 | 24.1 | 7.8 | 87.3 | 32.4 | 28.3 | 57000 | 3% | 7 | n | Neg | Neg | Pos | Neg | post hemolytic |

A- Age; S - Sex; T- Total count;, DC - Differential count;, RBC - Red blood cell; PCV - Packed cell volume; HB - Hemoglobin; MCV - Mean corpuscular MCHC - Mean corpuscular Hemoglobin concentration; RDW - Red cell distrubution width; RC - Reticulocyte count; OFT - Osmotic fragility test; HSBN - Hemosiderinuria, HBU - Hemoglobinuria; HS - Heriditary spherocytosis; Thal- Thalassaemia; HDN - Hemolytic disease of new born, HS - Heriditary spherocytosis; Thal Inter - Thalassaemia Intermedia; Neg - Negative; POS - Positive, D - Direct; I.D - Indirect